glycol, crosslinker, or combination thereof.

[0025] When the linker is a particle, in some embodiments, the particle can be modified by any methods known in the art. For example, the particle can be coated with streptavidin or a derivative thereof. In some embodiments, the particles can be modified or functionalized with at least one functional group. Examples of the functional groups can include, but are not limited to, amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups and any combination thereof.

[0026] The probe reagent of the detection reagent can be any targeting molecule of interest. Examples of the probe reagent can include, but are not limited to, a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, a virus, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a lipid, a glycoprotein, a peptidoglycan, an aptamer, and any combinations thereof. In some embodiments, the probe reagent can be modified by any means known to one of ordinary skill in the art. By way of example, the probe reagent can be genetically modified, or it can be biotinylated.

[0027] The nucleic acid label of the detection reagent can have any configuration and/or any sequence length. In some embodiments, the nucleic acid label can be single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer (e.g., a concatemer with a 3D structure such as a rolony, i.e., rolling-circle colony, or a DNA nanoball), or any combinations thereof. In various embodiments, the nucleic acid label can be designed for minimal cross-hybridization of bases with each other.

[0028] In some embodiments, the nucleic acid label can be a modified nucleic acid label. An exemplary modification of the nucleic acid label includes, without limitations, conjugation of the nucleic acid label to one or more detectable molecules. The detectable molecule can include any optical molecule, including, but not limited to, a small-molecule dye, a fluorescent protein, a quantum dot, or any combinations thereof.

[0029] In some embodiments, the nucleic acid label can comprise at least a partially double-stranded region. For example, the nucleic acid label can be pre-hybridized with at least one optically-labeled decoder probe, e.g., to produce at least the first signal of the temporal image stack. In such embodiments, additional decoder probes can be added during the detection method described herein to hybridize with other single-strand subsequences of the nucleic acid label.

[0030] In some embodiments, the nucleic acid label can comprise a plurality of pre-

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determined subsequences. Each of the pre-determined subsequences can be independently of any length. In some embodiments, at least one of the pre-determined subsequences can comprise one or more nucleobases. In certain embodiments, at least one of the pre-determined subsequences can comprise from 1 to 100 nucleobases.

[0031] The pre-determined subsequences with the nucleic acid label can be conjugated together by at least one sequence linker. In some embodiments, the sequence linker can be a direct bond, e.g., a phosphoester bond, which can allow conjugation of the pre-determined subsequences to form a longer, contiguous sequence. In some embodiments, the sequence linker can be a nucleotidic linker. When the pre-determined subsequences are conjugated together by a nucleotidic linker, the nucleotidic linker can have a sequence length of at least one nucleotide. The nucleotidic linker, in some embodiments, can be single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

[0032] Depending on various applications and/or assay conditions (e.g., sensitivity, sample volume/concentration), a detection signal of a probe can be amplified by conjugating the probe to a plurality of the nucleic acid labels. In such embodiments, the detection reagent can comprise one probe reagent and a plurality of nucleic acid labels. Without wishing to be limiting, in other embodiments, the detection reagent can comprise a plurality of probe reagents and a nucleic acid label. In some embodiments, the detection reagent can comprise a plurality of probe reagents and a plurality of nucleic acid labels.

[0033] The detection reagents and methods described herein can be used in any biological assays for detection, identification and/or quantification of target molecules or analytes in a sample. In particular embodiments, the detection reagent can be present in a soluble phase for various biological assays. By way of example only, in some embodiments, the detection reagent can be adapted for use in immunofluorescence. For example, the detection reagent adapted for use in immunofluorescence can be used to identify microbes or pathogens. In alternative embodiments, the detection reagent can be adapted for use in immunohistochemistry. For example, the detection reagent adapted for use in immunohistochemistry can be used to study tissue biopsies or cultured cells. In some embodiments, the detection reagent and the method described herein can be applied to fixed cells and/or living cells. In other embodiments, the detection reagent can be adapted for use in fluorescence in situ hybridization. In some embodiments, the detection reagent can be adapted for use in western blot. Accordingly, the

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detection reagent described herein can be adapted for use in various applications, e.g., but not limited to, pathogen detection and/or identification, cancer-tissue analysis and other medical pathology applications, lineage tracking of differentiating stem cells, and lineage tracking and/or identification of dendritic cells.

[0034] Kits for various biological assays also provided herein. In some embodiments, a kit can comprise: (a) a plurality of the detection reagents described herein or a portion thereof; and (b) at least one reagent. Examples of a reagent include, but are not limited to, a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

[0035] The kits provided herein can be used for sequencing-based readout or hybridization-based readout. In some embodiments where the kit is used for hybridization-based readout, the kit can further comprise at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

[0036] In some embodiments, the kit can comprise a plurality of at least one component of the detection reagents, e.g., the "nucleic acid label" component of the detection reagents. In such embodiments, users can attach the provided nucleic acid labels to their probe reagents of interest to form their own detection reagents described herein. In such embodiments, the kit can further comprising at least one coupling agent that allows the user to conjugate at least one nucleic acid label to the user's probe reagents of interest. In other embodiments, the nucleic acid labels can be already attached to the pre-determined probe reagents and are thus provided to users in the form of the detection reagents that are ready to use.

[0037] In some embodiments, the detection reagents provided in the kit or formed by a user can be provided in a solution phase. In other embodiments, the detection reagents provided in the kit or formed by a user can be immobilized in a multi-well plate.

[0038] In some embodiments of any aspects described herein, the analytes or target molecules can be present in a solution phase. In some embodiments, the analytes or target molecules can be immobilized on a solid substrate or support.

[0039] To clarify, the compositions and methods described herein are different from the ones described in the US Patent Application No.: US 2007/0231824. The '824 application discusses methods of decoding a sensor array containing immobilized microspheres, wherein the

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microspheres are immobilized on a solid support (e.g., an array substrate), rather than designed to be in a solution phase. As such, a sample fluid is flowed over the sensor array containing immobilized microspheres. The analytes in the sample fluid then bind to the immobilized microspheres. After binding, the sample fluid is then discarded and the immobilized microspheres are analyzed. Accordingly, the compositions and the methods described in the '824 application cannot be used and detected directly on a sample (e.g., on a tissue sample) or in situ as described herein, e.g., immunofluorescence, immunohistochemistry, fluorescence in situ hybridization, or western blot.

[0040] Further, the assay methods described herein are also different from the general nanostring technology or other technologies as described in the U.S. Patent No.: US 7,473,767, and the U.S. Patent Application No.: US 2010/0047924. The general nanostring technology and nanoreporter detection methods described in the '924 application are based on determination of the "spatial location of signals" emanating from the labeled nanostrings or nanoreporters. The detection methods described in the '767 patent is based on the color resulting from various ratios of different optical labels bound to the polynucleotide probes. All these previous methods will require at least a plurality of optical labels to be detected simultaneously for determination of spatial location of signals or the resultant color from various ratios of different optical labels. Accordingly, all these previous methods do not involve detection of signals in a temporally-sequential manner as described herein. Further, there are at least two disadvantages of nanostring technology based on detection of "spatial location of signals," rather than temporal detection of signals as described herein. First, compared to the methods and detection reagents described herein, the nanostring technology generally requires very high optical magnification for spatially discerning separation of colors that are typically located very close to each other within a nanostring; thus limiting a field of view/sample size, and precision, and/or increasing instrument cost. Second, unlike the methods and detection reagents described herein, the nanostring technology generally requires a thorough control of the amount of probes used in detection, because too few probes would yield a signal that is difficult to be detected, but too many probes would increase the likelihood of probes overlapping, and thus making the readout impossible.

BRIEF DESCRIPTION OF THE DRAWINGS

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[0041] **Fig. 1** shows three different embodiments of the detection reagents described herein producing distinct sequencing readout. Pathogen-specific antibodies (e.g., anti-E-Coli, anti-S. aureus, and anti-*C. albicans*) are individually conjugated to a nanoparticle with at least one nucleic acid label as described herein. In accordance with one or more embodiments, the readout of the nucleic acid label can take the form of a set of optical images or spot-readings of, e.g., fluorescent or visible colors; the temporal sequence of optical images or spot-readings can then be computationally analyzed to determine the identity of the corresponding probe reagent, e.g., a pathogen-specific antibody.

[0042] **Fig. 2** shows three different embodiments of the detection reagents described herein producing distinct sequencing readout. DNA oligonucleotides complementary to target RNA expression (e.g., OCT4, SOX2, or KLF4) are individually conjugated to a nanoparticle with at least one nucleic acid label as described herein. In accordance with one or more embodiments, the readout of the nucleic acid label can take the form of a set of optical images or spot-readings of, e.g., fluorescent or visible colors; the temporal sequence of optical images or spot-readings can then be computationally analyzed to determine the identity of the corresponding probe reagent, e.g., a DNA aptamer specific for a RNA expression.

[0043] **Fig. 3** shows exemplary forms of a nucleic acid label of the detection reagent according to one or more embodiments described herein.

[0044] **Figs. 4A** and **4B** shows two exemplary configurations of probe reagents and nucleic acid labels on particles, in accordance with one or more embodiments described herein.

[0045] **Fig. 5** shows one embodiment of the detection reagents for an exemplary hybridization-based readout method, in accordance with one or more embodiments described herein. Pathogen-specific antibodies (e.g., anti-*C. albicans*) are individually conjugated to a nanoparticle comprising at least one nucleic acid label as described herein. In accordance with one or more embodiments, the readout of the nucleic acid label can be determined by hybridizing it with a small number of, e.g., fluorescently-labeled decoder probes, imaging, and then advancing to the next set of decoder probes. In order to allow the SeqTag to be read out quickly and without the use of enzymes or chemical reactions, the DNA oligonucleotide (SeqTag) is designed to include several hybridization sites, each corresponding to a particular readout step. At each readout step, the sample is subjected to a mixture of fluorescently labeled DNA probes that could potentially bind that step's hybridization site. Each of the sites, however, is designed

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to bind only one of these probes, thus revealing the SeqTag's identifying code.

[0046] **Figs. 6A-6B** show the readout images of DYNABEADS® beads (1 μm in size) localized on a sample using an exemplary hybridization-based detection method. Each bead was conjugated to one of 6 nucleic acid labels, which in turn hybridized with a different set of decoder probes conjugated to either a green, red or blank optical label during each readout stage (**Fig. 6A**: Readout stage 1; **Fig. 6B**: Readout stage 2).

[0047] **Fig. 7** is a set of images showing that each of the detection molecule constructs (SeqTag labels) properly stained the yeast and fluorescenced in accordance with three pre-determined sets of decoder probes. SeqTag labels and oligonucleotide displacers were applied as follow:

Step 1: Set 1 Probes only;

Step 2: Set 1 Displacement oligonucleotides and Set 2 Probes;

Step 3: Set 2 Displacement oligonucleotides and Set 3 Probes; and

Step 4: Set 3 Displacement oligonucleotides.

SeqTag Label and corresponding colors were as follow:

Set 1: SeqTag 1 - Green, SeqTag 2 - Red, and SeqTag 3 - Blue;

Set 2: SeqTag 4 - Green, SeqTag 5 - Red, and SeqTag 6 - Blue;

Set 3: SeqTag 7 - Green and SeqTag 8 - Red.

[0048] **Fig. 8** shows SeqTag readout by a displacement-hybridization experiment according to an embodiment of the method described herein. SeqTag DNA labels were incubated on a streptavidin-coated microarray. This microarray was then exposed to a sequence of fluorescently labeled detection probes and displacement oligonucleotidess, as per displacement-hybridization readout. Imaging the array after each readout step demonstrated fluorescence corresponding to the expected pattern (shown next to each image).

[0049] **Fig. 9** is a schematic representation of displacement hybridization according to an embodiment of the method. As the readout progresses, fluorescence from prior readout steps needst be removed so as not to obstruct the current step's signal. As illustrated in **Fig. 9**, this can be accomplished using a displacement-hybridization method: each of the SeqTag's hybridization sites can be preceded by a short "toehold" sequence. During each readout step, the sample is subjected to a mixture of "displacer" DNA oligonucleotides. One of these displacers is

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complementary to the preceding step's hybridization site and, with the help of the toehold, is capable of displacing the fluorescent probe that was bound there.

[0050] **Fig. 10** is a schematic representation of a probe reagent according to an embodiment described herein. Shown is an oligonucleotide-antibody-streptavidin construct. A convenient and effective way to SeqTag-label antibodies is through a streptavidin bridge. The antibody is biotinylated and the probe DNA oligonucleotides (SeqTag) are synthesized with a 5'-biotin modification. Then, by taking advantage of streptavidin' s native tetrameric form, three DNA strands are bound to each antibody. As opposed to chemical conjugation methods, which can harm the antibody, this method proves gentle enough to preserve antibody function. As shown a single streptavidin molecule (2) is bound to three of the same DNA SeqTags (1) and a single antibody (3).

[0051] **Fig. 11** is a schematic representation of detection of an analyte by the SeqTag labeled antibody shown in **Fig. 10**.

[0052] **Fig. 12** is a schematic representation of detection reagents for different analytes. As shown, different infectious agent (analytes) can each be assigned their own SeqTag code to enable multiplexed detection according to an embodiment described herein.

[0053] **Fig. 13** is a schematic representation of detection reagents for SeqTagged Fluorescence *in situ* hybridization (FISH). FISH permits microbes to be identified based on their ribosomal RNA sequence. In the case of SeqTagged FISH, the FISH probe and its SeqTag can be synthesized as a single DNA oligonucleotides as shown. Sequences shown are, from top to bottom, SEQ ID NO: 1 (5'-CCTACACACCAGCGTGCC-3', probe for *K. pneumonia*); SEQ ID NO: 2 (5'-CCGCACTTTCATCTTCCG-3', probe for *H. influenza*); and SEQ ID NO: 3 (GCCAAGGCTTATACTCGC, probe for *C. albicans*).

DETAILED DESCRIPTION OF THE INVENTION

[0054] Described herein are methods, detection reagents (or detection molecules as used interchangeably herein) and kits for detecting a plurality of analytes in a sample. In accordance with embodiments of various aspects described herein, a probe reagent (e.g., antibody or aptamers) can be directly or indirectly labeled with a nucleic acid label. The nucleic acid information present on the nucleic acid label can then be decoded and/or detected in a temporally-sequential manner. The detection reagents and methods described herein significantly

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increase the number of different probes (and corresponding analytes) that can be simultaneously detected in a multiplex assay, as compared to an traditional assay where each probe is labeled with only fluorescent labels or quantum dots, and thus multiplexing is limited by the number of available and practically usable colors. Furthermore, because the detection reagents described herein are detected and/or imaged in a temporal series of steps, the number of probes (and corresponding analytes) that can be detected in a multiplex assay grows multiplicatively with the number of detection steps in a time series and the number of optical labels being used. By way of example only, 3 set of images in which 4 distinct optical labels are used can encode 4 x 4 x 4 = 64 distinct probe reagents (e.g., antibodies).

Methods of detecting a plurality of analytes in a sample

[0055] One aspect of the inventions provides the methods for detecting a plurality of analytes in a sample, using the detection reagents described herein. The method includes (a) contacting the sample with a composition comprising a plurality of detection reagents (which will be described in detail later), wherein each subpopulation of the detection reagents targets at least one different analyte; and (b) detecting in a temporally-sequential manner said plurality of the pre-determined subsequences of said detection reagents, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents. In some embodiments, the signal signature is a temporal signature. In some embodiments, the signal signature can further comprise a spatial signature. A non-limiting example of a spatial signature includes spatial location of a signal signature.

[0056] In some embodiments, the temporal order of the signal signatures corresponding to the plurality of the subsequences of the detection reagent can be unique for each subpopulation of the detection reagents. In some embodiments, at least two or more signal signatures can be used to identify the same subpopulation of the detection reagents.

[0057] In some embodiments, a detection reagent described herein can target at least two (e.g., at least two, at least three or more) distinct analytes. In some embodiments, a first subpopulation of the detection reagents can target at least one analyte different from that of a second subpopulation of the detection reagents. By way of example only, a first subpopulation of

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the detection reagents can target at least analyte A and analyte B, whereas a second subpopulation of the detection reagents can target at least analyate B and analyte C. The readout of these detection reagents can be distinct but overlapping. Thus, different analytes can be identified by sampling them combinatorially and determining which one binds.

[0058] As used herein, the term "temporal order of the signal signatures" refers to a sequence of signal signatures determined in a temporally-sequential manner, i.e., the sequence of signal signatures is progressed through by a number of active operations performed in a temporally-sequential manner, e.g., using a different set of decoding reagents or decoder probes in each active operation. In some embodiments, using a set of decoder probes in each active operation can yield one signal signature corresponding to one subsequence of the detection reagents.

[0059] The composition comprising a plurality of detection reagents can exist in any format. In some embodiments, the composition comprising a plurality of detection reagents can be in a form of a solution or suspension comprising the detection reagents. In such embodiments, the composition can further comprise at least one agent. For example, without wishing to be bound, the agent can be a blocking buffer, a surfactant, unconjugated probe reagents, a stabilizer, an enzyme inhibitor, or any combinations thereof. In some embodiments where the compositions are administered *in vivo*, the composition can further comprise a pharmaceutically-acceptable carrier. In other embodiments, the composition comprising a plurality of detection reagents can be contained or immobilized in a device (e.g., a syringe, or a microfluidic device) or an assay or reaction vessel (e.g., solid supports such as vials, and multi-well plates).

[0060] As used therein, the term "contacting" refers to any suitable means for delivering, or exposing, a sample to a plurality of the detection reagents described herein. In some embodiments, the term "contacting" refers to adding the detection reagents (e.g., suspended in a solution) directly to the sample. In some embodiments, the term "contacting" can further comprise mixing the sample with the detection reagents by any means known in the art (e.g., vortexing, pipetting, and/or agitating). In some embodiments, the term "contacting" can further comprise incubating the sample together with the detection reagents for a sufficient amount of time, e.g., to allow binding of the probe reagents to the target analytes. The contact time can be of any length, depending on the binding affinities and/or concentrations of the probe reagents and/or the analytes, concentrations of the detection reagents, and/or incubation condition (e.g., temperature). For example, the contact time can be reduced if the sample and detection reagents

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are incubated at a higher temperature. In some embodiments, the contact time between the sample and the detection reagents can be at least about 30 seconds, at least about 1 minute, at least about 5 minutes, at least about 10 minutes, at least about 15 minutes, at least about 30 minutes, at least about 1 hour, at least about 2 hours, at least about 3 hours, at least about 4 hours, at least about 6 hours, at least about 8 hours, at least about 10 hours, at least about 12 hours, at least about 24 hours, at least about 48 hours or longer. One of skill in the art can adjust the contact time accordingly.

[0061] For in vivo applications, the term "contacting" can refer to administering the detection reagents to a subject, e.g., by oral administration or by injection.

[0062] The sample can be contacted with at least one kind of the detection reagents. In some embodiments, the sample can be contacted with at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 or more different kinds of the detection reagents. In some embodiments, the sample can be contacted with at least 100, at least 500, at least 1000, at least 5000, at least 10,000, at least 50,000, at least 100,000 or more different kinds of the detection reagents. Various kinds of the detection reagents described herein can differ in types of probe reagents (e.g., nucleic acids vs. antibodies), target binding domains, and/or target analytes.

[0063] In some embodiments, the method described herein can further comprise processing the sample before contacting with the composition comprising a plurality of detection reagents described herein. Depending on the types and/or natures of the samples and/or analytes, different sample processing techniques can be used with the methods described herein. Exemplary sample processing techniques include, but are not limited to, mechanical processing of a sample (e.g., without limitations, homogenizing, centrifuging, vortexing, sectioning and shearing), addition of at least one reagent to a sample (e.g., without limitations, lysis buffers, RNA or DNA extraction reagents, RNA or DNA digestion reagents, enzyme inhibitors, fixing agents, organic solvents, antibodies, permeabilizing agents and immunohistochemistry agents), separation of a sample (e.g., without limitations, filtering, centrifuging, electrophoresis, western blot, and Northern blot), mounting a sample on a solid support (e.g., a microscopic slide), and any combinations thereof.

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[0064] By way of example only, if a sample is a tissue from a subject (e.g., a biopsy for immunostaining), sample processing can include, but are not limited to, tissue sectioning, mounting on a solid support, fixing the tissue, permeabilizing the tissue (if intracellular proteins are to be detected), blocking non-specific reactions with the detection reagents. In some embodiments, proteins or nucleic acids can be isolated from a tissue or fluid sample and then separated electrophoretically on a separation medium (e.g., electrophoresis gel), followed by transferring the proteins or nucleic acids to a blotting membrane. The blotting membranes containing proteins or nucleic acids can then be contacted with the detection reagents described herein. Methods of processing samples before addition of various types of probe reagents for different kinds of assays are well established in the art, and any of those methods can be performed prior to the contacting step of the methods described herein.

[0065] In some embodiments, the method described herein can further comprise removing any unbound detection reagents before detection of the pre-determined subsequences in a temporally-sequential manner. The term "unbound detection reagents" as used herein refers to detection reagents that have not bound to or interacted with target analytes. The unbound detection reagents can be removed from the sample by any methods known in the art, e.g., rinsing with the sample with a buffered solution at least once, at least two times, at least three times or above.

[0066] After the detection reagents bind to the target analytes in a sample, the nucleic acid labels of the detection reagents carrying nucleic-acid information can be decoded to allow identification of respective probe reagent(s) conjugated to them, as opposed to traditional optical labeling technologies, where an optical signature such as a fluorophore is detected in the absence of providing any nucleic acid information. In embodiments of various aspects described herein, the pre-determined subsequences within the nucleic acid labels are detected in a temporally-sequential manner. The term "temporally-sequential manner" is used in reference to detecting or decoding in a time series a plurality of the pre-determined subsequences within the nucleic acid labels of any detection reagents that are bound to target analytes in a sample. In some embodiments, one or more pre-determined subsequences within at least one nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of a time series. In some embodiments, one pre-determined subsequence within at least one nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of

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a time series. In some embodiments, at least one pre-determined subsequence (e.g., 1, 2, 3, 4, 5, 6, or more pre-determined subsequences) at the same corresponding location within the nucleic acid label of each detection reagent can be detected or decoded at each time point or detection step of a time series. The time period between any two time points or detection steps can be of any length, e.g., seconds, minutes and hours. For example, the time period between any two time points or detection steps can vary from about 5 seconds to about 2 hours, from about 10 seconds to about 1 hour, from about 30 seconds to about 30 mins, or from about 1 min to about 15 mins. In some embodiments, the time period between any two time points or detection steps can be less than 5 seconds. In other embodiments, the time period between any two time points or detection steps can be longer than 2 hours, longer than 4 hours, longer than 6 hours, longer than 12 hours, longer than 1 day. For example, a sample containing the detection reagents can be maintained at room temperature, at a fridge temperature (e.g., between about 0 °C and about 10°C) or at sub-zero temperatures (e.g., between -80°C or lower and 0°C) during the time period between detection steps. In some embodiments, each subsequent detection step is performed substantially immediately one after another (e.g., within less than 2 seconds, less than 1 second).

[0067] In some embodiments, the pre-determined subsequences can be detected in any temporal orders. In some embodiments, the next pre-determined subsequence to be detected after the previous one can be located closest to the previous one. In some embodiments, the next pre-determined subsequence to be detected after the previous one can be located at least one, at least two, at least three, at least four, at least five or more pre-determined subsequences apart from the previous one. In such embodiments, any pre-determined subsequences that were bypassed in a previous detection step can be detected afterward. In some embodiments of the methods described herein, a computer-implemented software can be used to facilitate an analysis of the temporal readouts from the detection steps, e.g., re-arranging the temporal readouts in an order corresponding to their spatial locations within the nucleic acid label before further comparison and quantification analyses.

[0068] In some embodiments, the detection or decoding of the pre-determined subsequences can comprise nucleic acid sequencing. Methods for sequencing nucleic acids are well established to a skilled artisan, e.g., but not limited to ligation, hybridization, synthesis, amplification or single-base extension, or any combinations thereof. By way of example only, as shown in **Fig. 1** or **Fig. 2**, the nucleic acid labels each contain three pre-determined subsequences (each of one

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nucleotide) conjugated together by a direct bond such as a phosphodiester bond. Each sequencing step decodes or determines one nucleotide, wherein each nucleobase (A, G, C or T) generates a distinct signal signature corresponding to the nucleobase. Consequently, a temporal order or time series of the signal signatures generated from each sequencing step corresponds to the respective probe reagent, and thus identify the target analyte. Without wishing to be bound, in this embodiment, the number of sequencing steps performed is not necessarily equal to the number of the pre-determined sequences. In some embodiments, the number of sequencing steps performed can be less than the number of the pre-determined sequences. For example, as shown in **Fig. 1**, two sequencing steps could be sufficient to identify the three different probe reagents, where each base is associated with a different color. However, additional nucleic acid information can increase the accuracy of identifying different probe reagents. Further, if the sequencing of each base can yield one of 4 colors, the n-base subsequences (e.g., 3-base subsequences shown in **Fig. 1** or **Fig. 2**) can produce 4^n possible unique readouts, i.e., 4^n possible distinct probe reagents can be distinguished using such detection reagents described herein.

[0069] While sequencing methods can convey single base difference, other detection or decoding methods that convey information by the presence or absence of entire hybridization "sites" or pre-determined subsequences on the nucleic acid label can also be used for the methods described herein. In some embodiments, the detection step can comprise hybridizing a decoder probe with a subsequence on the nucleic acid label of the detection reagent, wherein the decoder probe can comprise a detectable label. In particular embodiments, the detection method can comprise: (a) hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes can comprise a detectable label, each detectable label producing a signal signature; (b) detecting said signal signature produced by the hybridization of said set of decoder probes; and (d) repeating steps (a) and (b) for other subsequences of said detection reagents.

[0070] In some embodiments, each subpopulation of the decoder probes can comprise a different detectable label, each different detectable label producing a different signal signature. In these embodiments, the different signal signature produced by the hybridization of the set of decoder probes can be detected.

[0071] In some embodiments, each subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the subsequence

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of the detection reagents. In some embodiments, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to distinct subsequences of the detection reagents. In some embodiments, at least two or more subpopulations of the decoder probes can bind to the same subsequence of the detection reagents. For example, a first subpopulation and a second subpopulation of the decoder probes can be complementary (e.g., partially complementary or completely complementary) to the same subsequence of the detection reagents.

[0072] By way of example only, **Fig. 5** shows an exemplary detection reagent comprising anti-*C. albicans* probe reagents and nucleic acid labels containing three hybridization sites or pre-determined subsequences conjugated together by sequence linkers. In the first hybridization step, a first set of decoder probes each comprising a distinct detectable label (e.g., complementary DNA readout probes shown in **Fig. 5**, each comprising a distinct optical label) is hybridized with a first pre-determined subsequence (e.g., Site 1 in **Fig. 5**), followed by detection of a first signal signature produced by the hybridization. In the second hybridization step, a second set of decoder probes each comprising a distinct detectable label is hybridized with a second pre-determined subsequence (e.g., Site 2 in **Fig. 5**), followed by detection of a second signal signature produced by the hybridization. The second pre-determined subsequence can be the same or different from the first pre-determined subsequence. However, in preferred embodiments, the second pre-determined subsequence is different from the first pre-determined subsequence, e.g., to minimize cross-hybridization with each other. Accordingly, the hybridization and signal detection steps are repeated for other subsequences of the detection reagents with a different set of decoder probes, thereby producing a temporal order or time series of the signal signatures corresponding to the respective probe reagent (and detection reagent).

[0073] As used herein, the term "decoder probe" refers an oligonucleotide with a sequence complementary to a pre-determined sequence of the nucleic acid label. By "complementary" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non- traditional types. The decoder probe sequence can be completely or partially complementary to a pre-determined sequence. In some embodiments, partial complementarity is indicated by the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9,10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100%

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complementary). "completely complementary" or 100% complementarity means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. Less than perfect complementarity refers to the situation in which some, but not all, nucleoside units of two strands can hydrogen bond with each other.

[0074] The decoder probe can have a sequence of any length. In some embodiments, the decoder probe can have a sequence length of about 1 to about 100 nucleotides, about 1 to about 50 nucleotides, about 2 to about 50 nucleotides, about 5 to about 30 nucleotides, or about 5 to about 20 nucleotides.

[0075] In some embodiments, the decoder probe can comprise at least one detectable label described herein. In some embodiments, the detectable label can be an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule or protein, a quantum dot, a colorimetric reagent, a chromogenic molecule or protein, a Raman label, and any combinations thereof. In some embodiments, the detectable label or optical label can be a fluorescent molecule or protein.

[0076] In some embodiments, the decoder probe can be modified, e.g., base modification or activated with a functional group for linkage to a detectable label.

[0077] The number of decoder probes in each set can vary, depending on the number of distinct subsequences in each hybridization. In some embodiments, there can be about 1 to about 100 decoder probes, about 2 to about 50 decoder probes, about 4 to about 20 decoder probes in each set. In some embodiments, there can be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50 or more decoder probes in each set. In some embodiments, while each subsequence site can hybridize with a large number of decoder probes, each *set* of decoder probes added in each readout step to hybridize with the subsequence site within the detection reagents can generally have as many as the number of available fluorescent colors. For example, each set of the decoder probes added in each readout step to hybridize with the subsequence site of the detection reagents can contain about 3-4 decoder probes, each of which is labeled with a distinct fluorescent color. In the case of using quantum dots or Raman labels as detection labels, there can be more than 3-4 decoder probes in each set added during each readout step.

[0078] Without wishing to be bound, an example of “non-overlapping nucleic acid labels” or “non-overlapping SeqTag labels” (i.e., no two decoder probes will hybridize with the same

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spatial site of the nucleic acid label) is shown herein for illustrative purposes. Assuming there are 12 pre-determined subsequences (e.g., nucleic acid sequences) designed for minimal cross-hybridization with each other and each others' complements (e.g., A1, A2, A3, A4, B1, B2, B3, B4, C1, C2, C3, and C4). Consider a detection reagent comprising a nucleic acid label of the form:

5' ------A[1-4]----B[1-4]----C[1-4]---- 3'

where each position (e.g., A[1-4]) holds only one of the subsequences (e.g., A2). Now, this subsequence is decoded or detected by using decoder probes or complementary probes A1* - A4* each labeled in one of four fluorescent colors. After readout, the signal produced by the fluorophore can be removed (e.g., denaturing to undo the hybridization (and thus removing the fluorophore)) before continuing with B1* - B4*. Since each step yields one of four outcomes, this coding scheme can provide 4 x 4 x 4 = 64 unique readouts. These hybridization-based probes can be used to label 64 different probe reagents that can be read out in three cycles.

[0079] In the case where two decoder probes can overlap, a single site (e.g., A in the above nucleic acid label form), which can accept one of the different decoder probes, can be used. This single site can be read out by subjecting it to the different decoder probes, e.g., in sets of 4 using the nucleic acid label form as shown above. When the correct set is reached, the nucleic acid label, e.g., SeqTag label, should be dark (no-color). This example is not construed to be limiting and any modifications apparent to one of skill in the art is also within the scope of the inventions.

[0080] The advantage of readout by hybridization is that it can be quick: hybridization can take place in minutes or less. Furthermore, no chemistry or enzymes are required during the readout process, as the readout process can be performed by similar methods as used in nucleic acid sequencing, microscopy, spectroscopy, or any combinations thereof. Thus, hybridization-based readout method can reduce cost, reduce reagent storage and/or simplify the process.

[0081] In some embodiments of the methods described herein, there can be no limit in the spatial movement of an analyte in a sample during a temporal detection of the detection reagents, for example, provided that the analyte stay within the field of detection and there is at least one same distinguishable feature in each image taken during a temporal detection so that the images can be aligned to each other based on the same distinguishable feature. In some embodiments where there is no such distinguishable feature, the spatial movement of an analyte in a sample can be less than 100 μm, including less than 50 μm, less than 25 μm, less than 10 μm, less than 1

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μm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. In some embodiments, the spatial movement of an analyte in a sample can be less than 1000 nm, including less than 500 nm, less than 250 nm, less than 100 nm, less than 50 nm, less than 10 nm or smaller, over a time period, during which a temporal detection of the detection reagents occurs. More importantly, the spatial movement limit of an analyte in a sample during a temporal detection is determined by the ability of matching distinguishable features between images taken during a temporal detection, which can be affected by imaging conditions. In some embodiments, the analyte can be fixed on a solid substrate or support. In some embodiments where there is or expects to be a spatial movement of an analyte during temporal detection, the location of the analyte with respect to a sample during each detection step can be determined and registered. Such spatial shift can then be corrected afterward during signal analysis using any art-recognized computer-implemented algorithms.

[0082] The length of time required to perform a temporal detection of the detection reagents (e.g., the length of time it takes to obtain a temporal sequence of signal signatures for the detection reagents) can vary, depending on the number of pre-determined subsequences to be detected or read and/or the number of available detection signals (e.g., fluorescent color and/or brightfield) to be read. In some embodiments, the length of time required to perform a temporal detection of the detection reagents can be, for example, but not limited to, 5 seconds, 10 seconds, 15 seconds, 20 seconds, 30 seconds, 1 mins, 2 mins, 3 mins, 4 mins, 5 mins, 15 mins, 30 mins, 1 hour, 2 hours, 4 hours, 6 hours, or longer.

[0083] The detection reagents can be detected by any means available in the art that is capable of detecting the specific signals on a given detection reagent generated during sequencing- or hybridization-based methods. Where the detection reagents (e.g., hybridized with decoder probes) are fluorescently labeled, suitable consideration of appropriate excitation sources can be readily determined. Possible sources can include but are not limited to arc lamp, xenon lamp, lasers, light emitting diodes or some combination thereof. The appropriate excitation source is used in conjunction with an appropriate optical detection system, for example an inverted fluorescent microscope, an epi-fluorescent microscope or a confocal microscope. Preferably, a microscope is used that can allow for detection with enough spatial resolution to separate distinct signals from individual detection reagents.

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[0084] Exemplary methods for detection of the detection reagents that are applicable to the methods described herein include, without limitations, the methods described in U.S. Pat. No. 7,473,767, US patent publication no. 2007/0166708, and US application number US 2010/0261026, all of which are incorporated by reference herein in its entirety.

[0085] Additional methods that can be used to detect optical signatures include, but are not limited to, any spectroscopic techniques, flow cytometry, or any art-recognized methods involving an optical scanner and/or a photodetector (e.g., without limitations, a charge-coupled devices, active pixel sensors, photodiode light sensors (e.g., LEDs), optical detectors, and any combinations thereof). Non-limiting examples of spectroscopic techniques can include absorption spectroscopy, emission spectroscopy, elastic scattering spectroscopy, reflection spectroscopy, impedance spectroscopy, inelastic spectroscopy, coherent or resonance spectroscopy, surface plasmon fluorescence spectroscopy, Raman spectroscopy, and any combinations thereof. Spectroscopy techniques can be used to detect light of any wavelengths, including, but not limited to, microwave, terahertz, infrared, near infrared, visible, ultraviolet, x-ray, gamma, and any combinations thereof.

[0086] Without wishing to be limited, in some embodiments, at least one pre-determined subsequence (e.g., individual bases or hybridization regions) can correspond to no optical signature; that is the absence of color can be considered as an additional color. In other embodiments, at least one pre-determined subsequence (e.g., individual bases or hybridization regions) can correspond to a compound optical signature, e.g., two or more simultaneous fluorescence in multiple channels during detection (e.g., by microscopy).

[0087] While a single area of a sample can interact with more than one probe reagents, the nucleic acid label can be designed such that any known or potential overlaps could be teased apart from the signal output. In the case where any probe may potentially overlap with all others, one can use the readout-by-hybridization variation and assign each probe a single unique hybridization sequence. Such approach can avoid multiple lengthy probe incubations and damaging stripping steps.

[0088] In some embodiments of the methods described herein, the signal signatures produced during any readout step (e.g., sequencing-based or hybridization-based) should be removed before advancing to the next pre-determined subsequence of the detection reagents. The removal of the signal signatures can be done by any methods known in the art, including, but not limited

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to, washing, heating, photo-bleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation, and any combinations thereof.

[0089] In some embodiments, the decoder probes can be designed such that they can be simply washed out either with a plain buffer, or they can be modified by varying salt concentrations or using detergents or denaturants such as formamide or dimethyl sulfoxide (DMSO).

[0090] In other embodiments, the fluorescence or color signature of a readout step can be attenuated or eliminated by photo-bleaching the signal using sufficient optical exposure. In alternative embodiments, the fluorescence or color signature of a readout step can be attenuated or eliminated by subjecting the fluorescence or color signature to chemical degradation under appropriate conditions, e.g., using a reducing agent or oxidizing solution such as 0.01 M sodium periodate.

[0091] In some embodiments, the decoder probes can be displaced from their hybridization sites by introducing other reagents or probe displacers that have stronger binding affinities to those same sites. This can be done, for example, by using nucleic acid sequences that are longer than and/or have better complementarity than the decoder probe sequences. For example, to create "better complementarity" of the probe displacers, in some embodiments, mismatches can be seeded in the hybridization region. In other embodiments, the hybridization region can be preceded and/or post-ceded with a "toe-hold" of around 3-8 bases (e.g., 6 bases). In such embodiments, the "better complementarity" of the probe displacer can be outside of the hybridization region, which can make the design of the hybridization regions easier.

[0092] In some embodiments, enzymes can be used to displace, digest, cut and/or cleave the detectable labels, the decoder probe sequence, the hybridized complex (formed by the decoder probe and pre-determined subsequence), and/or the cleavable sequence linker to the hybridized complex, in order to remove the signal signatures. One example is to introduce a deoxyuridine into the decoder probe. This modified base can be cleaved using the enzyme mix known as USER, thereby cutting the decoder probe sequence into two parts. Since each part is now shorter, it is characterized by a lower melting temperature and can melt off the hybridization sites of the detection reagents. Alternatively, one of skill in the art can employ one of numerous art-recognized sequence-specific nucleases or restriction enzymes, which can cut either the decoder probe sequence, the pre-determined subsequence that has been hybridized with decoder probes,

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the cleavable sequence linker attached to the hybridized subsequence and/or the hybridized complex thereof, thereby removing the signal signature.

[0093] In some embodiments, thermal denaturing can be used to remove the signal signature from a previous readout. In some embodiments where sequencing is involved, thermal denaturing can be reduced or avoided by using a sequencing-by-ligation approach and by setting the nucleic acid label base that immediately follows the sequencing primer (in the direction of ligation) to an adenine. Correspondingly, the ligation probes should then include a (deoxy-)uracil in the primer-proximal position. Without wishing to be bound by theory, an enzyme such as USER, which cleaves DNA at uracils can be used to remove the ligation probe and ready the system for the next sequencing step. These fragments will have lower melting temperatures than their parent probes, and these temperatures can be designed to fall below the operating temperature (or require an acceptable denaturing temperature).

[0094] After detection of the pre-determined subsequences is completed in a temporally-sequential manner, in some embodiments, the method described herein can further comprise comparing the temporal order of the signal signatures with different identifiers of said at least one probe reagent, wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample. In some embodiments, the method can further comprise measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents. In some embodiments, the intensity of the signal signatures generated from each subpopulation of the detection reagents can indicate an amount of the analyte. In some embodiments, the relative intensity of the signal signatures can be used in identification of each subpopulation of the detection reagents. Thus, the intensity of the signal signatures can be used as part of a coding scheme of the detection reagents described herein. The comparing and intensity measuring steps can be performed, e.g., by a computer-implemented software or algorithm.

[0095] Types of signal signature(s) can vary upon different embodiments of detection reagents and/or decoder probes described herein. As used herein, the term "signal signature" refers to a change in, or occurrence of, a response or indicator that is detectable either by observation or instrumentally. In certain instances, the signal signature is fluorescence or a change in fluorescence, e.g., a change in fluorescence intensity, fluorescence excitation or emission wavelength distribution, fluorescence lifetime, and/or fluorescence polarization. By

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way of example only, the fluorescence can be produced by binding a fluorophore to a decoder probe, and/or by detecting the hybridization using a fluorescent dye, e.g., SYBR Gold, that lights up when nucleic acid sequence becomes double-stranded. In certain other instances, the signal signature can be radioactivity (i.e., radiation), including alpha particles, beta particles, nucleons, electrons, positrons, neutrinos, and gamma rays emitted by a radioactive substance such as a radionuclide. By way of example, the detection reagents and/or decoder probes can comprise an optical molecule or label, thus producing optical signatures. Examples of optical signatures can include, without limitations, signatures of fluorescent color, visible light, no-color, and any combinations thereof. In such embodiments, the optical signatures can be detected by optical imaging or spectroscopy.

Detection reagents (or detection molecules as used interchangeably herein)

[0096] Another aspect provided herein is a detection reagent, which can be, for example, used in the methods described herein for any multiplexing assays. The detection reagent comprises at least one probe reagent and at least one nucleic acid label, wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner; wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

[0097] The detection reagents described herein can exist in different forms. By way of example only, in some embodiments, the detection reagent can be a detection molecule. In some embodiments, the detection reagent can be a detection particle. In some embodiments, the detection reagent can be multi-molecular.

[0098] As used herein, the term “conjugated" refers to two molecules being linked to each other, e.g., attaching a probe reagent to a nucleic acid label. The conjugation process can be performed, e.g., via a chemical reaction, or via a linker, which will be described later.

[0099] Depending on various applications and/or assay conditions (e.g., sensitivity, sample volume/concentration), a readout signal of a detection reagent can be amplified by increasing the number of the nucleic acid labels present in the detection reagent, e.g., by conjugating at least one probe reagent to a plurality of nucleic acid labels. In such embodiments, a plurality of the nucleic acid labels present in the detection reagent can range from about 2 to about 100,000,

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about 2 to about 10,000, about 2 to about 1,000, or about 2 to about 100. In some embodiments where the detection reagent comprises a particle as a hub, the number of possible nucleic acid labels present in the detection reagent can depend on the size of a particle. Generally, the larger the particle it is, the more nucleic acid labels can be incorporated into the detection reagent. For example, a particle of about 1-2 μm in size can allow incorporation of about 100,000 nucleic acid labels into the detection reagent. In some embodiments, there can be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 5000, 10000, 50000, 100000 nucleic acid labels present in the detection reagent. One of skill in the art can determine the optimum number of nucleic acid labels present the detection reagent without any undue experimentation.

[00100] The detection reagents described herein can be used in any biological assays for detection, identification and/or quantification of target molecules or analytes, including counting marked cells such as bacteria or cancer cells, in a sample. By way of example only, in some embodiments, the detection reagent can be adapted for use in immunofluorescence. In alternative embodiments, the detection reagent can be adapted for use in immunohistochemistry. In other embodiments, the detection reagent can be adapted for use in fluorescence in situ hybridization. In some embodiments, the detection reagent can be adapted for use in western blot. Depending on the nature of the sample and/or applications, the detection reagent can be adapted to be in any format, e.g., immobilized on a solid support, or in a solution or suspension phase. In certain embodiments, the detection reagent can be adapted to be present in a solution or suspension phase. The phrase "in a solution or suspension phase" as used herein generally refers to suspending the detection reagents in a liquid fluid, e.g., an aqueous buffer solution. Additional applications of the detection reagents and/or methods described herein will be discussed.

Probe reagents (or probe molecules as used interchangeably herein)

[00101] Each of the detection reagents described herein can comprise any number of probe reagents. In some embodiments, the detection reagent can comprise one or more probe reagents, e.g., at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 or more probe reagents. In one embodiment, the detection reagent can comprise one probe reagent. In other embodiments, the detection reagent can comprise a plurality of probe reagents, e.g., ranging from about 2 to about 100,000 probe reagents, about 2 to about 10,000 probe reagents, about 2 to about 1,000 probe reagents, or about 2 to about 100 probe reagents. In

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some embodiments where the detection reagent comprises a particle as a hub, the number of possible probe reagents present in the detection reagent can depend on the size of a particle. Generally, the larger the particle it is, the more probe reagents can be incorporated into the detection reagent. For example, a particle of about 1-2 μm in size can allow incorporation of about 100,000 probe reagents into the detection reagent. In some embodiments, there can be about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 50, 100, 500, 1000, 5000, 10000, 50000, 100000 probe reagents present in the detection reagent. One of skill in the art can determine the optimum number of probe reagents present the detection reagent without any undue experimentation.

[00102] As used interchangeably herein, the term "probe," "probe reagent" or "probe molecule" refers to an entity (e.g., but not limited to, a molecule, a particle, a composite entity, or a multi-molecular entity) that interacts with or binds to a target molecule or an analyte for the analysis of the target or the analyte. Typically the nature of the interaction or binding is noncovalent, e.g., by hydrogen, electrostatic, or van der Waals interactions, however, binding can also be covalent. Probe reagents can be entities (e.g., but not limited to, molecules, a particles, composite entities, or multi-molecular entities) capable of undergoing binding or molecular recognition events with target molecules. Probe reagents can be naturally-occurring, recombinant or synthetic. Examples of the probe reagent can include, but are not limited to, a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, an aptamer, and any combinations thereof. By way of example only, in immunohistochemistry, the probe reagent can include an antibody specific to the target antigen to be analyzed. An ordinary artisan can readily identify appropriate probe reagents for the target molecules or analytes of interest to be detected in various bioassays. In some embodiments, the probe reagent can be multi-molecular. For example, in one embodiment, the probe reagent can comprise a particle, an antibody, biotin and/or streptavidin, or any combinations thereof.

[00103] In some embodiments, the probe reagents can be modified by any means known to one of ordinary skill in the art. Methods to modify each type of probe reagents are well recognized in the art. Depending on the types of probe reagents, an exemplary modification includes, but is not limited to genetic modification, biotinylation, labeling (for detection purposes), chemical modification (e.g., to produce derivatives or fragments of the probe reagent),

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and any combinations thereof. In some embodiments, the probe reagent can be genetically modified. In some embodiments, the probe reagent can be biotinylated.

[00104] As used herein, the terms “proteins" and “peptides" are used interchangeably herein to designate a series of amino acid residues connected to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", and "peptide", which are used interchangeably herein, refer to a polymer of protein amino acids, including modified amino acids (e.g., phosphorylated, glycated, etc.) and amino acid analogs, regardless of its size or function. Although "protein" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term "peptide" as used herein refers to peptides, polypeptides, proteins and fragments of proteins, unless otherwise noted. The terms "protein" and "peptide" are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary peptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[00105] As used herein, the term “peptidomimetic” refers to a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide

[00106] The term “nucleic acids" used herein refers to polymers (polynucleotides) or oligomers (oligonucleotides) of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar linkages. The term “nucleic acid” also includes polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Exemplary nucleic acids include, but are not limited to, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), locked nucleic acid (LNA), peptide nucleic acids (PNA), and polymers thereof in either single- or double-stranded form. Locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo conformation. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. Such LNA oligomers are generally synthesized chemically. Peptide nucleic acid (PNA) is an artificially synthesized polymer similar to DNA or RNA. DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, whereas PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. PNA is generally synthesized chemically. Unless specifically limited, the term “nucleic

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acids" encompasses nucleic acids containing known analogs of natural nucleotides, which have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer, et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka, et al., J. Biol. Chem. 260:2605-2608 (1985), and Rossolini, et al., Mol. Cell. Probes 8:91-98 (1994)). The term "nucleic acid" should also be understood to include, as equivalents, derivatives, variants and analogs of either RNA or DNA made from nucleotide analogs, and, single (sense or antisense) and double-stranded polynucleotides.

[00107] In some embodiments, the term "nucleic acid" described herein can include a modified nucleic acid. Modified nucleic acids are well known in the art. Thus, a nucleic acid described herein can comprise one or more nucleic acid modifications known in the art. For example, the nucleic acid can comprise one or more nucleic acid modifications selected from the group consisting of internucleotide linkage modifications (intersugar linkage modifications), sugar modifications, nucleobase modifications, backbone modifications/replacements, and any combinations thereof. Exemplary internucleotide linkage modifications include, but are not limited to, phosphorothioate, phosphorodithioate, phosphotriester (e.g. alkyl phosphotriester), aminoalkylphosphotriester, alkyl-phosphonate (e.g., methyl-phosphonate), selenophosphate, phosphoramidate (e.g., N-alkylphosphoramidate), boranophosphonate, and the like. Exemplary sugar modifications include, but are not limited to, 2′-*O*-Me (2′-*O*-methyl), 2′-*O*-MOE (2′-*O*-methoxyethyl), 2'-F, 2′-*O*-[2-(methylamino)-2-oxoethyl] (2′-*O*-NMA), 2'-*S*-methyl, 2'-O-CH_2-(4'-C) (LNA), 2'-O-CH_2CH_2-(4'-C) (ENA), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), arabinose sugar, and the like. Exemplary nucleobase modifications include, but are not limited to, inosine, xanthine, hypoxanthine, nubularine, isoguanisine, tubercidine, 5-methylcytosine (5-me-C); 5-hydroxymethyl cytosine; xanthine; hypoxanthine; 2-aminoadenine; 6-methyl and other 6-alkyl derivatives of adenine and guanine; 2-propyl and other 2-alkyl derivatives of adenine and guanine; 2-thiouracil; 2-

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thiothymine; 2-thiocytosine; 5-propynyl uracil; 5-propynyl cytosine; 6-azouracil; 6-azocytosine; 6-azothymine; 5-uracil (pseudouracil); 4-thiouracil; 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines; 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines; 7-methyl and other 7-alkyl derivatives of adenine and guanine; 8-azaguanine; 8-azaadenine; 7-deazaguanine; 7-deazaadenine; 3-deazaguanine; 3-deazaadenin; universal base; and any combinations thereof. Exemplary backbone modifications include, but are not limited to, morpholino, cyclobutyl, pyrrolidine, peptide nucleic acid (PNA), aminoethylglycyl PNA (*aeg*PNA), backnone-extended pyrrolidine PNA (*bep*PNA), and the like.

[00108] The term "enzymes" as used here refers to a protein molecule that catalyzes chemical reactions of other substances without it being destroyed or substantially altered upon completion of the reactions. The term can include naturally occurring enzymes and bioengineered enzymes or mixtures thereof. Examples of enzyme families include kinases, dehydrogenases, oxidoreductases, GTPases, carboxyl transferases, acyl transferases, decarboxylases, transaminases, racemases, methyl transferases, formyl transferases, and α-ketodecarboxylases.

[00109] As used herein, the term "aptamers" means a single-stranded, partially single-stranded, partially double-stranded or double-stranded nucleotide sequence capable of specifically recognizing a selected non-oligonucleotide molecule or group of molecules. In some embodiments, the aptamer recognizes the non-oligonucleotide molecule or group of molecules by a mechanism other than Watson-Crick base pairing or triplex formation. Aptamers can include, without limitation, defined sequence segments and sequences comprising nucleotides, ribonucleotides, deoxyribonucleotides, nucleotide analogs, modified nucleotides and nucleotides comprising backbone modifications, branchpoints and nonnucleotide residues, groups or bridges. Methods for selecting aptamers for binding to a molecule are widely known in the art and easily accessible to one of ordinary skill in the art.

[00110] As used herein, the term "antibody" or "antibodies" refers to an intact immunoglobulin or to a monoclonal or polyclonal antigen-binding fragment with the Fc (crystallizable fragment) region or FcRn binding fragment of the Fc region. The term "antibodies" also includes "antibody-like molecules", such as fragments of the antibodies, e.g., antigen-binding fragments. Antigen-binding fragments can be produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. "Antigen-binding

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fragments" include, inter alia, Fab, Fab', F(ab')2, Fv, dAb, and complementarity determining region (CDR) fragments, single-chain antibodies (scFv), single domain antibodies, chimeric antibodies, diabodies, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen binding to the polypeptide. Linear antibodies are also included for the purposes described herein. The terms Fab, Fc, pFc', F(ab') 2 and Fv are employed with standard immunological meanings (Klein, Immunology (John Wiley, New York, N.Y., 1982); Clark, W. R. (1986) The Experimental Foundations of Modern Immunology (Wiley & Sons, Inc., New York); and Roitt, I. (1991) Essential Immunology, 7th Ed., (Blackwell Scientific Publications, Oxford)). Antibodies or antigen-binding fragments specific for various antigens are available commercially from vendors such as R&D Systems, BD Biosciences, e-Biosciences and Miltenyi, or can be raised against these cell-surface markers by methods known to those skilled in the art.

[00111] As used herein, the term "Complementarity Determining Regions" (CDRs; i.e. , CDR1, CDR2, and CDR3) refers to the amino acid residues of an antibody variable domain the presence of which are necessary for antigen binding. Each variable domain typically has three CDR regions identified as CDR1, CDR2 and CDR3. Each complementarity determining region may comprise amino acid residues from a "complementarity determining region" as defined by Kabat (i.e. about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al. , Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. about residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). In some instances, a complementarity determining region can include amino acids from both a CDR region defined according to Kabat and a hypervariable loop.

[00112] The expression "linear antibodies" refers to the antibodies described in Zapata et al. , Protein Eng., 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (VH -CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

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[00113] The expression "single-chain Fv" or "scFv" antibody fragments, as used herein, is intended to mean antibody fragments that comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. (Plöckthun, The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer- Verlag, New York, pp. 269-315 (1994)).

[00114] The term "diabodies," as used herein, refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) Connected to a light-chain variable domain (VL) in the same polypeptide chain (VH - VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. (EP 404,097; WO 93/11161; Hollinger et ah, Proc. Natl. Acad. Sd. USA, P0:6444-6448 (1993)).

[00115] As used herein, the term “small molecules" refers to natural or synthetic molecules including, but not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, aptamers, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[00116] The term “cells" used herein refers to any cell, prokaryotic or eukaryotic, including plant, yeast, worm, insect and mammalian. Mammalian cells include, without limitation; primate, human and a cell from any animal of interest, including without limitation; mouse, hamster, rabbit, dog, cat, domestic animals, such as equine, bovine, murine, ovine, canine, feline, etc. The cells may be a wide variety of tissue types without limitation such as; hematopoietic, neural, mesenchymal, cutaneous, mucosal, stromal, muscle spleen, reticuloendothelial, epithelial, endothelial, hepatic, kidney, gastrointestinal, pulmonary, T-cells etc. Stem cells, embryonic stem (ES) cells, ES- derived cells and stem cell progenitors are also included, including without

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limitation, hematopoeitic, neural, stromal, muscle, cardiovascular, hepatic, pulmonary, gastrointestinal stem cells, etc. Yeast cells may also be used as cells in some embodiments of the methods described herein. In some embodiments, the cells can be ex vivo or cultured cells, e.g. in vitro. For example, for ex vivo cells, cells can be obtained from a subject, where the subject is healthy and/or affected with a disease. Cells can be obtained, as a non-limiting example, by biopsy or other surgical means know to those skilled in the art.

[00117] As used herein, the term "antigens" refers to a molecule or a portion of a molecule capable of being bound by a selective binding agent, such as an antibody, and additionally capable of being used in an animal to elicit the production of antibodies capable of binding to an epitope of that antigen. An antigen may have one or more epitopes. The term "antigen" can also refer to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by MHC molecules. The term "antigen", as used herein, also encompasses T-cell epitopes. An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. This may, however, require that, at least in certain cases, the antigen contains or is linked to a Th cell epitope and is given in adjuvant. An antigen can have one or more epitopes (B- and T-epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be evoked by other antigens. Antigens as used herein may also be mixtures of several individual antigens.

[00118] In some embodiments, the probe reagent can be an antibody or a portion thereof, or an antibody-like molecule. In such embodiments, the probe reagents can be used to, for example, detect and/or identify pathogen type or speices, the presence of cell or disease markers, cellular protein expression levels, phosphorylation or other post-translation modification state, or any combinations thereof. By way of example only, **Fig. 1** shows three different embodiments of the detection reagents comprising at least one (e.g., 1, 2, 3, 4, 5 or more) pathogen-specific antibodies (e.g., anti-E. Coli, anti-S. aureus, and anti-C. albicans).

[00119] In some embodiments, the probe reagent can be a nucleic acid (e.g., DNA, RNA, LNA, PNA, or any combinations thereof). In such embodiments, the nucleic acids can be used to determine, for example, the existence of characteristic cellular DNA or RNA sequences (such as

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in fluorescent in situ hybridization), RNA expression levels, miRNA presence and expression, and any combinations thereof, in various applications, e.g., for pathogen detection and/or identification.

[00120] In some embodiments, the probe reagent can be a protein or a peptide. In such embodiments, the protein or peptide can be essentially any proteins with known binding targets. Examples include, but are not limited to, innate-immune proteins (e.g., without limitations, MBL, Dectin-1, TLR2, and TLR4 and any proteins disclosed in U.S. Provisional Application No.: 61/508,957, the content of which is incorporated herein by reference in its entirety) and proteins comprising the chitin-binding domain. Such innate-immune proteins and chitin-binding domain proteins can be used to detect their corresponding pattern-recognition targets (e.g., microbes such as bacteria) and fungus, respectively. By way of example only, instead of using pathogen-specific antibodies as probe reagents in the detection reagents as shown in **Fig. 1**, innate-immune proteins (e.g., MBL) or chitin-binding domain proteins can be used as probe reagents for detection of pathogens. While such detection reagents can be used to detect pathogens, they may not be pathogen-specific, as compared to the ones using pathogen-specific antibodies as probes molecules.

[00121] In some embodiments, the probe reagent can be an aptamer. In some embodiments, the probe reagent can be a DNA or RNA aptamer. The aptamers can be used in various bioassays, e.g., in the same way as antibodies or nucleic acids described herein. By way of example only, **Fig. 2** shows some exemplary embodiments of the detection reagents comprising at least one (e.g., 1, 2, 3, 4, 5 or more) DNA aptamers (e.g., with a nucleotide sequence complementary to nuclear reprogramming factors, such as Oct4, Sox2, and Klf4). Such detection reagents can be used to determine RNA expression level of nuclear reprogramming factors in somatic cells for detecting, screening, or identifying stem cells (e.g., induced pluripotency stem cells).

[00122] In some embodiments, the probe reagent can be a cell surface receptor ligand. As used herein, a "cell surface receptor ligand" refers to a molecule that can bind to the outer surface of a cell. Exemplary, cell surface receptor ligand includes, for example, a cell surface receptor binding peptide, a cell surface receptor binding glycopeptide, a cell surface receptor binding protein, a cell surface receptor binding glycoprotein, a cell surface receptor binding organic compound, and a cell surface receptor binding drug. Additional cell surface receptor

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ligands include, but are not limited to, cytokines, growth factors, hormones, antibodies, and angiogenic factors.

[00123] When the detection reagents described herein are used as targeted delivery vehicles, e.g., for a diagnostic agent, in some embodiments, the probe reagent can be an endosomolytic ligand. As used herein, the term "endosomolytic ligand" refers to molecules having endosomolytic properties. Endosomolytic ligands can promote the lysis of and/or transport of the composition described herein, or its components, from the cellular compartments such as the endosome, lysosome, endoplasmic reticulum (ER), Golgi apparatus, microtubule, peroxisome, or other vesicular bodies within the cell, to the cytoplasm of the cell. Some exemplary endosomolytic ligands include, but are not limited to, imidazoles, poly or oligoimidazoles, linear or branched polyethyleneimines (PEIs), linear and branched polyamines, e.g. spermine, cationic linear and branched polyamines, polycarboxylates, polycations, masked oligo or poly cations or anions, acetals, polyacetals, ketals/polyketals, orthoesters, linear or branched polymers with masked or unmasked cationic or anionic charges, dendrimers with masked or unmasked cationic or anionic charges, polyanionic peptides, polyanionic peptidomimetics, pH-sensitive peptides, natural and synthetic fusogenic lipids, natural and synthetic cationic lipids.

[00124] In other embodiments, the probe reagent for use in delivery of an agent (e.g., a diagnostic agent) encapsulated within the detection reagents described herein can be a PK modulating ligand. As used herein, the terms "PK modulating ligand" and "PK modulator" refers to molecules which can modulate the pharmacokinetics of the composition described herein. Some exemplary PK modulator include, but are not limited to, lipophilic molecules, bile acids, sterols, phospholipid analogues, peptides, protein binding agents, vitamins, fatty acids, phenoxazine, aspirin, naproxen, ibuprofen, suprofen, ketoprofen, (S)-(+)-pranoprofen, carprofen, PEGs, biotin, and transthyretia-binding ligands (e.g., tetraiidothyroacetic acid, 2, 4, 6-triiodophenol and flufenamic acid).

[00125] In various embodiments, the detection reagent described herein can comprise one kind/species of probe reagents or different kinds/species of probe reagents. In some embodiments, the kind/species of the probe reagents present in the detection reagent can be the same. In other embodiments, the detection reagent can include at least one different kind/species of the probe reagents (e.g., 1, 2, 3, 4, 5, or 6 probe reagent species). In such embodiments, the

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distinct probe reagent species can be different from the others by types (e.g., antibodies vs. DNA aptamers), binding domains, and/or target analytes.

Nucleic acid labels

[00126] In accordance with embodiments of various aspects described herein, the nucleic acid label or nucleic acid tag comprises at least one pre-determined nucleic acid subsequence, which is used to identify an analyte or target. In some embodiments, the nucleic acid label or nucleic acid tag can comprise any number of the pre-determined nucleic acid subsequences, e.g., ranging from about 1 to about 100, from about 2 to about 80, or from about 3 to about 50. In some embodiments, the nucleic acid label or nucleic acid tag can comprise at least about 1, at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 15, at least about 20, at least about 30, at least about 40, at least about 50, at least about 60, at least about 70 or more pre-determined nucleic acid subsequences. In some embodiments, the nucleic acid label or nucleic acid tag can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 70 or more pre-determined nucleic acid subsequences. Without wishing to be bound, the minimum number of pre-determined nucleic acid subsequences (n) required in the detection reagent can vary upon the number of distinct probes to be detected (X) and/or the number of distinct detectable labels (e.g., optical labels such as fluorescent labels or quantum dots) available to be used (Y), and n can be determined by the equation: $n = ceiling(\frac{\ln X}{\ln Y})$, where the mathematical function "ceiling" refers to rounding up a non-integer number to the nearest integer, when needed. For example, if 4 distinct detectable labels (Y) are used to distinguish 62 distinct probe reagents (X), n = ceiling (2.98) ~3. Therefore, at least three pre-determined nucleic acid subsequences are required in this example.

[00127] In some embodiments where the detectable labels include no-color (dark), the number of distinct detectable label available to be used can become Y+1. In such embodiments, n can be determined by the equation: $n = ceiling(\frac{\ln X}{\ln(Y+1)})$, and thus fewer pre-determined nucleic acid subsequences can be used. However, the combination in which all readout cycles are dark should not be allowed.

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[00128] Further, the pre-determined nucleic acid subsequences can be designed such that each readout cycle can light up in multiple colors. Thus, 2^Y instead of Y is used in the equation for n above and fewer pre-determined nucleic acid subsequences can thus be required. However, the combination in which all readout cycles are dark should not be allowed.

[00129] Additionally, while colors are generally used in a binary fashion, i.e., whether the color is present or not, in the above examples, the color intensity can also be used as a parameter of a signal signature. For example, if one detectable label is allowed to light up twice as brightly in a different color than another, the multiplexing capacity can be even further expanded.

[00130] The pre-determined nucleic acid subsequences can be constructed from any types of nucleic acids, including, but not limited to, DNA, RNA, PNA, LNA and any combinations thereof.

[00131] Each of the pre-determined subsequences of the nucleic acid label can be independently of any length. In certain embodiments, the pre-determined subsequences can each independently comprise a length of about 1 nucleobase to about 100 nucleobases, from about 1 nucleobase to about 50 nucleobases, from about 2 nucleobases to about 50 nucleobases, from about 5 nucleobases to about 30 nucleobases, or from about 5 nucleobases to about 20 nucleobases. In some embodiments, the pre-determined subsequences can each independently comprise one or more nucleobases, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleobases.

[00132] The achievable length of the pre-determined subsequences can affect the degree of multiplexibility. In some embodiments, the achievable length of the pre-determined subsequences can be a function of the decreasing fluorescence intensity over many cycles of stripped and rehybridization (i.e. diffusion of the sequenceable particles, degradation, increasing background noise). In the case of DNA or RNA based nucleic acid labels that are amplified in situ, modified base is incorporated during its synthesis (i.e., dUTP, biotin, Acrydite, aminoallele), enabling these detection reagents to be permanently embedded in a film (regardless of the film thickness) of functionalized polyacrylamide (i.e. Acrydite streptavidin, NHS ester Acrydite). The embedded material can then be stripped and rehybridized for many more cycles without altering its spatial architecture and minimizing the background noise.

[00133] Two or more pre-determined subsequences can be conjugated together within a nucleic acid label using any methods known in the art. In some embodiments, two or more pre-

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determined subsequences can be conjugated together by a sequence linker. The term "sequence linker" as used herein generally refers to an entity that connects two sequences or subsequences as described herein together.

[00134] In some embodiments, the sequence linker can be a direct bond or an atom such as nitrogen, oxygen or sulfur; a unit such as NR_1, C(O), C(O)NH, SO, SO_2, SO_2NH; or a chain of atoms. If needed, the two ends of the pre-determined subsequences can be linked together by providing on the two ends of the pre-determined subsequences complementary chemical functionalities that undergo a coupling reaction. In particular embodiments, the sequence linker is a direct bond, including, but not limited to, a phosphodiester bond. For example, a 3' carbon atom of a sugar base at the 5' end nucleotide of a first pre-determined subsequence can interact with the 5' carbon atom of another sugar base at the 3' end nucleotide of a second pre-determined subsequence to form a covalent bond, e.g., a phosphodiester bond. As such, two or more pre-determined subsequences can be bonded together to form a longer and contiguous pre-determined subsequence.

[00135] In some embodiments, the sequence linker can be a nucleotidic linker. The term "nucleotidic linker" as used herein refers to a linker of one nucleotide long or a sequence substantially comprising a plurality of nucleotides. In some embodiments, the nucleotidic linker can have a sequence length of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20, at least 30 or more nucleotides. The sequence length of the nucleotidic linker can vary with a number of factors, e.g., detection methods, and/or properties of optical labels. Without wishing to be bound by theory, in some embodiments, increasing the length of the nucleotidic linker can increase the flexibility of the nucleic acid label, e.g., to increase the binding frequency between a pre-determined sequence and a decoder probe, the term of which will be discussed later. However, too long a nucleotidic linker can result in a too long nucleic acid label, which could overlap with other nucleic acid labels of the detection reagents during an assay and thus reduce the quality and/or accuracy of the signal detection. One of skill in the art can determine the optimum length of the nucleotidic linker without undue experimentations.

[00136] The nucleotidic linker can be in any structure or conformation. In some embodiments, the nucleotidic linker can be in a structure selected from the group consisting of single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

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[00137] In some embodiments, the sequence linker can be a bead or a nanoparticle acting as a hub. Accordingly, two or more pre-determined subsequences can be independently conjugated together via a bead or a nanoparticle.

[00138] Without wishing to be bound, while the sequence linker can be a direct bond, an atom, a nucleotidic linker, or any combinations thereof, the sequence linker can also include a sequence of amino acids, a polymer chain, a microbead, a nanobead, or any combinations thereof. To provide for the linkages between sequence linker, in some embodiments, different functionalities can be introduced to the ends of sequence linker and/or the pre-determined subsequences. Examples of functionalities include, but are not limited to, amide groups, including carbonic acid derivatives, ethers, esters, including organic and inorganic esters, amino, urethane, urea and any combinations thereof.

[00139] In some embodiments, the nucleic acid label is substantially a polynucleotide sequence containing one or more pre-determined subsequences. In such embodiments, any two pre-determined subsequences are either joined or conjugated together by a direct bond such as a phosphodiester bond (to produce longer contiguous subsequence), a nucleotidic linker of any desirable length, or any combinations thereof.

[00140] In such embodiments, the nucleic acid label of the detection reagent can be adapted to any configuration or structure. In some embodiments, the nucleic acid label can be single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof. In some embodiments, the nucleic acid label can be a linear polynucleotide sequence.

[00141] In some embodiments, the nucleic acid label can be a circular polynucleotide sequence. The advantage of using a circular nucleic acid label is that it can be amplified using rolling-circle amplification or hyperbranched rolling-circle amplification to generate a long continuous nucleic acid molecule that contains multiple copies of the same nucleic acid label sequences linked in series (also known as concatemer), thus resulting in an amplified signal. In some embodiments, instead of pre-forming the detection reagents comprising the circular nucleic acid label(s) and the probe reagent(s), detection reagents comprising linear polynucleotide(s) and the probe reagent(s) can be first synthesized. The circular nucleic acid labels can then added to hybridize with the linear polynucleotide(s) before or after the probe reagent(s) bind to the analytes. In such embodiments, while requiring an extra step, this approach can have the

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advantage over direct attachment of circular nucleic acid labels in that it does not require chemical modification of the nucleic acid label for conjugation with the probe reagents, thus resulting in a circular nucleic acid label that is compatible with a broader range of amplification enzymes. Furthermore, the linear polynucleotides can be smaller than the secondary circular nucleic acid labels, facilitating diffusion of the probe reagents (and the detection reagents) to their targets. In other embodiments, the linear polynucleotides can be circularized using a suitable double-stranded or single-stranded ligase, with or without the addition of a suitable ligation template. Without wishing to be bound by theory, such embodiments can avoid the extra hybridization that is required when a pre-circularized oligonucleotide is used instead.

[00142] When the detection reagents described herein are used as FISH probes, the nucleic acid labels and the FISH probes can be part of the same nucleic acid sequence construct, and thus the circularization can encompass the entire construct (e.g., both the nucleic acid labels and FISH probes). A schematic representation of exemplary FISH probes for SeqTagged FISH is shown in **Fig. 13**.

[00143] In some embodiments, the method described herein can be used to identify a class an analyte, e.g., a pathogen belongs to. For example, the method can be used to identify if a pathogen is a Gram-negative, Gram-positive, or some other class of pathogen, e.g., yeast. Thus, the method described herein can be used to as Gram test to determine whether a suspected pathogen is Gram positive, Gram negative or yeast. This can be useful for quickly identifying the type of infection in a subject and administering appropriate therapy. By way of example only, this can be accomplished using a detection reagent comprising a class-specific probe, also referred to as "Gram-stain like probe" herein. Again by way of example only, the probe can be a DNA probe for FISH.

[00144] In some embodiment, the FISH probe for identifying eubacteria can comprise the nucleotide sequence of SEQ ID NO: 4 (GCTGCCTCCCGTAGGAGT). An exemplary SeqTag labeled FISH-probe for identifying eubacteria can comprise the nucleotide sequence of SEQ ID NO: 5 (CTGCCTCCCGTAGGAGTTTTTTCGCTTTAGCCTAAGTGAAATC).

[00145] In some embodiments, the FISH probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 6 (CTCTGGCTTCACCCTATTC. An exemplary SeqTag labeled FISH-probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 7 (CTCTGGCTTCACCCTATTCTTTTTCGCTTTTTTGGGGAAAAGACA).

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[00146] In some embodiments, the FISH probe for identifying firmicutes can comprise the nucleotide sequence of SEQ ID NO: 8 (CGGAAGATTCCCTACTGC). An exemplary SeqTag labeled FISH-probe for identifying yeast can comprise the nucleotide sequence of SEQ ID NO: 9 (CGGAAGATTCCCTACTGCTTTTTCGCTTTCTGTAATGGAGTGGA).

[00147] In the SeqTag labeled FISH probes discussed above, each probe can be assigned a particular "signal signature" for each of the three readout steps. For example, colors can be labeled at B, C, and D. In some embodiments, each color can be from a different fluorophore, such as FAM, Cy3 and Cy5. Each signal signature can take advantage of multiple fluorophores simultaneously or even the same fluorophore multiple times, e.g., a SeqTag with signal signature corresponding to "DDDC."

[00148] An exemplary signal signature for identifying eubacteria, yeast or firmicutes is shown in **Table 1**. As shown, each class has a different assigned code for the first readout step. For example, for the first readout, eubacteria are assigned color C' yeast color D and firmicutes color B. On readout, eubacteria will show up as color C, yeast as color D, firmicutes as both colors B and C.

Table 1

Name	Assigned code				Effective code			
	S1	S2	S3		S1	S2	S3	
Eubacteria-Kempf	C				C			
All_yeast-Kempf	D				D			
Firmicutes-pB-00196	B				BC			

[00149] After identifying the class of pathogens, the pathogens can be further probed for identifying the specific pathogen or genus using the method described herein. For example, the detection reagent can comprise a pathogen specific probe that binds to a specific pathogen or genus of pathogens. For example, the probe can be a FISH probe that specifically binds to a specific pathogen. Some exemplary pathogen specific FISH probes are shown in **Table 2**.

Table 2

Name		probeBase FISH Sequence	Assigned code		Effective code	
SEQ ID NO:	Gram +					
10	Staph_spp-Kempf	TCCTCCATATCTCTGCGC	DD	B	BC DD	B
11	S_Aureus-Kempf	GAAGCAAGCTTCTCGTCCG	CD	B	BC CDDD	BB
12	Streptococcus_spp-	CACTCTCCCCTTCTGCAC	DD	D	BC DD	D

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	Kempf							
13	S_Pneumoniae-Kempf	GTGATGCAAGTGCACCTT	B	DC		BC	DDB	DDC
14	S_Pyogenes-Kempf	TTCCAAAGCGTACATTGGTT	C	DB		BC	DDC	DDB
15	S_Agalactiae-Kempf	GTAAACACCAAACMTCAGCG	D	DC		BC	DDD	DDC
16	B_subtilis-pB-00401	CGA AGG GGA CGT CCT ATC T	C	DD		BC	C	DD
17	L_acidophilus-pB-00711	CAG GCT TGC TCC TCG TTG	DD	C		BC	DD	C
	Gram -							
18	P_Aeruginosa-Kempf	TCTCGGCCTTGAAACCCC	B	B		C	B	B
19	K_Pneumoniae-Kempf	CCTACACACCAGCGTGCC	B	DD		C	B	DD
20	H_influenzae-pB-00348	CCG CAC TTT CAT CTT CCG	B	BC		C	B	BC
21	B_cepacia-pB-00346	CTG TGC GCC GGT TCT CTT	DD	B		C	DD	B
22	K_oxytoca-pB-01681	CTA CAA GAC TCC AGC CTG CC	DD	C		C	DD	C
23	E_coli-pB-02569-compl	ATG AGC AAA GGT ATT AAC TTT ACT CCC	B	C		C	B	C
24	Shewanella_spp-pB-01191-mod	AGC TAA TCC CAC CTA GGT TCA TC	BC	B		C	BC	B
25	H_pylori-pB-00361	CACACCTGACTGACTATCCCG	C	B		C	C	B
	Yeast							
26	C_Albicans-Kempf	GCCAAGGCTTATACTCGCT	C	BC		D	C	BC
27	C_Glabrata_Kempf	CCGCCAAGCCACAAGGACT	C	CD		D	C	CD
28	C_Krusei-Kempf	GATTCTCGGCCCCATGGG	BC	C		D	BC	C
29	C_Parapsilosis-Kempf	CCTGGTTCGCCAAAAAGGC	CD	C		D	CD	C

[00150] Exemplary SeqTag labeled FISH-probe for identifying a specific pathogen are shown in **Table 3**. As shown in **Table 3**, each specific pathogen can be assigned a specific signal signature based on an assigned color for each readout step and identity of a pathogen can be decoded using this table when the method is carried out using this set of assigned coding.

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Table 3

SEQ ID NO:	Name	SeqTag-labeled FISH-probe Sequence
		Gram +
30	Staph_spp-Kempf	TCCTCCATATCTCTGCGCTTTTTCGCTTTCTGGAGAAAGGGCCATTTT TCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTCTGGAGAAAGGGCC A
31	S_Aureus-Kempf	GAAGCAAGCTTCTCGTCCGTTTTTCGCTTTCTGGAGAAAGGGCCATT TTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTGGAAGCACCTAT TCC
32	Streptococcus_spp-Kempf	CACTCTCCCCTTCTGCACTTTTTCGCTTTCTGGAGAAAGGGCCATTTT TCGCTTTTCACGATCCCATGTATTTTTCGCTTTCTGGAGAAAGGGCC A
33	S_Pneumoniae-Kempf	GTGATGCAAGTGCACCTTTTTTTTCGCTTTGAAGCCGGTTATAGCTTT TTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTTCACGATCCCATGT A
34	S_Pyogenes-Kempf	TTCCAAAGCGTACATTGGTTTTTTTTCGCTTTCGGTTCCAAAGACACT TTTTCGCTTTGGAAGCACCTATTCCTTTTTCGCTTTTCACGATCCCAT GTA
35	S_Agalactiae-Kempf	GTAAACACCAAACMTCAGCGTTTTTCGCTTTGAAGCCGGTTATAGC TTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTTCACGATCCC ATGTA
36	B_subtilis-pB-00401	CGA AGG GGA CGT CCT ATC TTTTTTCGCTTTTCACGATCCCATGTATTTTTCGCTTTGGAAGCACCT ATTCCTTTTTCGCTTTTCACGATCCCATGTA
37	L_acidophilus-pB-00711	CAG GCT TGC TCC TCG TTGTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTGAAGCCG GTTATAGCTTTTTCGCTTTCTGGAGAAAGGGCCA
		Gram -
38	P_Aeruginosa-Kempf	TCTCGGCCTTGAAACCCCTTTTTCGCTTTTAGGCATTAGCATTGTTTT TCGCTTTCGGTTCCAAAGACAC
39	K_Pneumoniae-Kempf	CCTACACACCAGCGTGCCTTTTTCGCTTTTCACGATCCCATGTATTTT TCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTTCACGATCCCATGTA
40	H_influenzae-pB-00348	CCG CAC TTT CAT CTT CCGTTTTTCGCTTTCGGTTCCAAAGACACTTTTTCGCTTTTAGGCATT AGCATTGTTTTTCGCTTTGAAGCCGGTTATAGC
41	B_cepacia-pB-00346	CTG TGC GCC GGT TCT CTTTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTCGGTTCCA AAGACACTTTTTCGCTTTCTGGAGAAAGGGCCA
42	K_oxytoca-pB-01681	CTA CAA GAC TCC AGC CTG CCTTTTTCGCTTTCTGGAGAAAGGGCCATTTTTCGCTTTGAAGCCGG TTATAGCTTTTTCGCTTTCTGGAGAAAGGGCCA
43	E_coli-pB-02569-compl	ATG AGC AAA GGT ATT AAC TTT ACT CCCTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTGAAGCCGG TTATAGC
44	Shewanella_spp-pB-01191-mod	AGC TAA TCC CAC CTA GGT TCA TCTTTTTCGCTTTTAGGCATTAGCATTGTTTTTCGCTTTCGGTTCCAA AGACACTTTTTCGCTTTGGAAGCACCTATTCC

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45	H_pylori-pB-00361	CACACCTGACTGACTATCCCGTTTTTCGCTTTGGAAGCACCTATTCC TTTTTCGCTTTCGGTTCCAAAGACAC
	Yeast	
46	C_Albicans-Kempf	GCCAAGGCTTATACTCGCTTTTTTCGCTTTCGGTTCCAAAGACACTT TTTCGCTTTGGAAGCACCTATTCCTTTTTCGCTTTGAAGCCGGTTATA GC
47	C_Glabrata_Kempf	CCGCCAAGCCACAAGGACTTTTTTCGCTTTGAAGCCGGTTATAGCTT TTTCGCTTTGGAAGCACCTATTCCTTTTTCGCTTTTCACGATCCCATG TA
48	C_Krusei-Kempf	GATTCTCGGCCCCATGGGTTTTTCGCTTTTAGGCATTAGCATTGTTTT TCGCTTTGAAGCCGGTTATAGCTTTTTCGCTTTGGAAGCACCTATTC C
49	C_Parapsilosis-Kempf	CCTGGTTCGCCAAAAAGGCTTTTTCGCTTTCTGGAGAAAGGGCCATT TTTCGCTTTGAAGCCGGTTATAGCTTTTTCGCTTTGGAAGCACCTAT TCC

[00151] As shown in **Table 3**, the "signal signature" can comprise multiple fluorescence "colors" in each step. For example, *E. coli* can be marked as red in step 1 and red+green in step 2 and blue in step 3. The "signal signature" can also be encoded in the brightness of the color at each step. For example, *E. coli* could be marked as red in step 1 and 3 times brighter red in step 2.

[00152] Exemplary hybridization sites for generating the different signal signatures at different steps using the exemplary SeqTag labeled FISH-probes described above are shown in **Table 4**.

Table 4

SEQ ID NO:	**Assigned "Color"**	
		Set1
50	B	CGCTTTCTGTAATGGAGTGGA
51	C	CGCTTTAGCCTAAGTGAAATC
52	D	CGCTTTTTTGGGGAAAAGACA
		Set 2
53	B	CGCTTTTAGGCATTAGCATTG
54	C	CGCTTTGGAAGCACCTATTCC
55	D	CGCTTTCTGGAGAAAGGGCCA
		Set 3
56	B	CGCTTTCGGTTCCAAAGACAC
57	C	CGCTTTGAAGCCGGTTATAGC
58	D	CGCTTTTCACGATCCCATGTA

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[00153] In some embodiments, multiple detection reagents can be used to identify a specific analyte. For example, a first set of probes can be used to identify whether the potential pathogen is gram-position, gram-negative and then another set of probes to identify it more specifically. Both probes would need to produce a detectable signal, allowing one to use, for example, **Table 3** to uniquely identify the pathogen.

[00154] In some embodiments, the nucleic acid label can be a concatemer, including a rolony or a DNA nanoball that is large enough to act as a particle rather than simply a strand of nucleic acid. Using a concatemer as a nucleic acid label can eliminate the need for in-situ enzymatic treatment of circular nucleic acid amplification, but it can also increase the overall molecular weight of the detection reagent and thus retard diffusion. Similar to the circular nucleic acid labels as described above, an exemplary approach of hybridizing concatemers with the linear polypeptides of the detection reagents after probe reagents bind to the analytes can be used to facilitate diffusion of the probe reagents (and the detection reagents) to their targets.

[00155] To increase the accuracy and/or specificity of the methods described herein, in various embodiments, the nucleic acid label can be designed for minimal cross-hybridization of bases with each other. Various art-recognized computational programs or algorithms are available to design nucleic acid sequences with minimal cross-hybridization. Thus, one of skill in the art can optimize the nucleic acid label sequence using any methods or algorithms known in the art.

[00156] In some embodiments, the nucleic acid labels described herein can be synthetic nucleic acid molecules (e.g., DNA, RNA, or DNA/RNA hybrids), and can be rationally-designed to have features that optimize labeling and detection of the detection reagents, and that prevent secondary structure formation. In some embodiments, a nucleic acid label is a designed polynucleotide sequence from about 50 to 50,000 bases long.

[00157] In some embodiments, the nucleic acid labels described herein can be designed to minimize predictable secondary structures, and/or be designed such that each nucleic acid label can hybridize only against its own target. In some embodiments, the nucleic acid labels described herein can be designed to be devoid of any secondary structure. Putative secondary structures (e.g. hairpins, folding, or internal base pairing) can be predicted by methods known in the art such as MFOLD. Without intending to be limited to any theory, in some embodiments, predictable secondary structure in the nucleic acid label can be minimized by avoiding inverted

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repeats and by skewing the backbone-specific content such that the backbone of the nucleic acid label is CT or GA-rich. Any art-recognized methods, e.g., MFOLD, can be used to verify if each nucleic acid label can hybridize only against its own target.

[00158] Sequences can also be screened to avoid common six-base-cutter restriction enzyme recognition sites. Selected sequences can be additionally subjected to predicted secondary structure analysis, and those with the least secondary structure may be chosen for further evaluation. Any program known in the art can be used to predict secondary structure, such as the MFOLD program (Zuker, 2003, Nucleic Acids Res. 31 (13):3406-15; Mathews et al., 1999, J. Mol. Biol. 288:911-940).

[00159] In some embodiments, the nucleic acid label can comprise only a subset of the A, G, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only one of the A, G, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid can comprise two of the A, C, T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only three of the A, G, C, and T (and/or U) nucleotides or modified nucleotides thereof. In some embodiments, the nucleic acid label can comprise only four of the A, G, C, and T (and/or U) nucleotides or modified nucleotides thereof. The subset of the A, G, C and T (and/or U) nucleotides or modified nucleotide thereof can be selected from the group consisting of A; G; C; T; U; (A,G); (A,C); (G,T); (G,U); (C,T); (C,U); (G,T,U); (C,T,U); (A,C,G); (A,G,T); (A,G,U); (A,C,T); (A,C,U); (C,G,T); (C,G,U); (A,C,T,U); and (C,G,T,U).

[00160] Without wishing to be bound, the nucleic acid label can be a modified nucleic acid label. An exemplary modification of the nucleic acid label includes, without limitations, attaching one or more detectable molecules to the nucleic acid label (either to one end of or along the nucleic acid label sequence). The detectable molecule can be any optical molecule, including, but not limited to, a small-molecule dye, a fluorescent protein, a quantum dot, or any combinations thereof. In another embodiment, at least one end of the nucleic acid label can be modified to include a chemical functional group and/or a protein or peptide to facilitate the conjugation between the nucleic acid label and the probe reagent.

[00161] In some embodiments, at least one (including, e.g., at least two, at least three, at least four, at least five, at least six or more) nucleic acids or nucleotides present in the nucleic acid label can be modified. For example, the nucleic acid can comprise one or more nucleic acid

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modifications as described herein, e.g., selected from the group consisting of internucleotide linkage modifications (intersugar linkage modifications), sugar modifications, nucleobase modifications, backbone modifications/replacements, and any combinations thereof.

Conjugation between a nucleic acid label and a probe reagent

[00162] In accordance with embodiments of various aspects described herein, the detection reagent comprises at least one probe reagent and at least one nucleic acid label, wherein the probe reagent and the nucleic acid label can be conjugated together by any methods known in the art.

[00163] In some embodiments, the probe reagent and the nucleic acid label can be attached or conjugated together by a linker. As used herein, the term "linker" generally refers to an entity that connects the probe reagent and the nucleic acid label together. The linker can be monovalent or multivalent. The term "monovalent" as used herein refers to the capacity of a linker to join one probe reagent to one nucleic acid label. The term "multivalent" as used herein refers to the capacity of a linker to bind with one or more probe reagents and/or nucleic acid labels. In some embodiments, a multivalent linker can join at least one probe reagent to a plurality of nucleic acid labels (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleic acid labels).

[00164] In some embodiments, the term "linker" means an organic moiety that connects two parts of a compound. Such linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NH, C(O), C(O)NH, SO, SO_2, SO_2NH, SS, or a chain of atoms, such as substituted or unsubstituted C_1-C_6 alkyl, substituted or unsubstituted C_2-C_6 alkenyl, substituted or unsubstituted C_2-C_6 alkynyl, substituted or unsubstituted C_6-C_{12} aryl, substituted or unsubstituted C_5-C_{12} heteroaryl, substituted or unsubstituted C_5-C_{12} heterocyclyl, substituted or unsubstituted C_3-C_{12} cycloalkyl, where one or more methylenes can be interrupted or terminated by O, S, S(O), SO_2, NH, C(O).

[00165] In some embodiments, the linker is a branched linker. The branchpoint of the branched linker may be at least trivalent, but can be a tetravalent, pentavalent or hexavalent atom, or a group presenting such multiple valencies. In some embodiments, the branchpoint is -N, -N(R)-C, -O-C, -S-C, -SS-C, -C(O)N(R)-C, -OC(O)N(R)-C, -N(R)C(O)-C, or -N(R)C(O)O-C; wherein R is independently for each occurrence H or optionally substituted alkyl. In some embodiments, the branchpoint is glycerol or derivative thereof.

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[00166] In some embodiments, linker comprises a cleavable linking group. As used herein, a "cleavable linking group" is a chemical moiety which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least 10 times or more, preferably at least 100 times faster in the target cell or under a first reference condition (which can, e.g., be selected to mimic or represent intracellular conditions) than in the blood or serum of a subject, or under a second reference condition (which can, e.g., be selected to mimic or represent conditions found in the blood or serum).

[00167] Cleavable linking groups are susceptible to cleavage agents, e.g., pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, e.g., oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; amidases; endosomes or agents that can create an acidic environment, e.g., those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific) and proteases, and phosphatases.

[00168] A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted. For example, for liver targeting, cleavable linking groups can include an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

[00169] Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

[00170] In some embodiments, cleavable linking group is cleaved at least 1.25, 1.5, 1.75, 2, 3, 4, 5, 10, 25, 50, or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood or serum (or under in vitro conditions selected to mimic extracellular conditions). In some embodiments, the cleavable linking group is cleaved by less than 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, or 1% in the blood (or in

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vitro conditions selected to mimic extracellular conditions) as compared to in the cell (or under in vitro conditions selected to mimic intracellular conditions).

[00171] Exemplary cleavable linking groups include, but are not limited to, redox cleavable linking groups (e.g., -S-S- and $-C(R)_2$-S-S-, wherein R is H or C_1-C_6 alkyl and at least one R is C_1-C_6 alkyl such as CH_3 or CH_2CH_3); phosphate-based cleavable linking groups (e.g., -O-P(O)(OR)-O-, -O-P(S)(OR)-O-, -O-P(S)(SR)-O-, -S-P(O)(OR)-O-, -O-P(O)(OR)-S-, -S-P(O)(OR)-S-, -O-P(S)(ORk)-S-, -S-P(S)(OR)-O-, -O-P(O)(R)-O-, -O-P(S)(R)-O-, -S-P(O)(R)-O-, -S-P(S)(R)-O-, -S-P(O)(R)-S-, -O-P(S)(R)-S-, . -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -S-P(O)(H)-S-, and -O-P(S)(H)-S-, wherein R is optionally substituted linear or branched C_1-C_{10} alkyl); acid celavable linking groups (e.g., hydrazones, esters, and esters of amino acids, -C=NN- and -OC(O)-); ester-based cleavable linking groups (e.g., -C(O)O-); peptide-based cleavable linking groups, (e.g., linking groups that are cleaved by enzymes such as peptidases and proteases in cells, e.g., – $NHCHR^AC(O)NHCHR^BC(O)$-, where R^A and R^B are the R groups of the two adjacent amino acids). A peptide based cleavable linking group comprises two or more amino acids. In some embodiments, the peptide-based cleavage linkage comprises the amino acid sequence that is the substrate for a peptidase or a protease found in cells.

[00172] In some embodiments, an acid cleavable linking group is cleaveable in an acidic environment with a pH of about 6.5 or lower (e.g., about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid.

[00173] In addition to covalent linkages, two parts of a compound can be linked together by an affinity binding pair. The term "affinity binding pair" or "binding pair" refers to first and second molecules that specifically bind to each other. One member of the binding pair is conjugated with first part to be linked while the second member is conjugated with the second part to be linked. As used herein, the term "specific binding" refers to binding of the first member of the binding pair to the second member of the binding pair with greater affinity and specificity than to other molecules.

[00174] Exemplary binding pairs include any haptenic or antigenic compound in combination with a corresponding antibody or binding portion or fragment thereof (e.g., digoxigenin and anti-digoxigenin; mouse immunoglobulin and goat antimouse immunoglobulin) and

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nonimmunological binding pairs (e.g., biotin-avidin, biotin-streptavidin, biotin-neutravidin, hormone [e.g., thyroxine and cortisol-hormone binding protein, receptor-receptor agonist, receptor-receptor antagonist (e.g., acetylcholine receptor-acetylcholine or an analog thereof), IgG-protein A, IgG-protein G, IgG-synthesized protein AG, lectin-carbohydrate, enzyme-enzyme cofactor, enzyme-enzyme inhibitor, and complementary oligonucleotide pairs capable of forming nucleic acid duplexes), and the like. The binding pair can also include a first molecule which is negatively charged and a second molecule which is positively charged.

[00175] One example of using binding pair conjugation is the biotin-avidin, biotin-streptavidin or biotin-neutravidin conjugation. In this approach, one of the molecule or the peptide is biotinylated and the other is conjugated with avidin or streptavidin. Many commercial kits are also available for biotinylating molecules, such as proteins.

[00176] Another example of using binding pair conjugation is the biotin-sandwich method. *See, e.g.*, example Davis et al., Proc. Natl. Acad. Sci. USA, 103: 8155-60 (2006). The two molecules to be conjugated together are biotinylated and then conjugated together using at least one tetravalent avidin-like molecule (e.g., avidin, streptavidin, or neutravidin) as a linker.

[00177] Accordingly, in some embodiments, both the nucleic acid label(s) and probe reagent(s) can be biotinylated and then linked together using an avidin-like molecule (e.g., avidin, streptavidin, or neutravidin). In one embodiment, neutravidin and/or streptavidin is used as a linker to bridge together the biotinylated nucleic acid label(s), e.g., DNA sequence(s), and biotinylated probe reagent(s), e.g., antibody. Without wishing to be bound by theory, each avidin-like molecule (e.g., avidin, streptavidin, or neutravidin) generally has four binding sites, so at most four molecules can be linked together. For example, one biotinylated probe reagent can be linked, via an avidin-like molecule (e.g., avidin, streptavidin or neutravidin), to three biotinylated nucleic acid labels, or in any other combinations (e.g., two biotinylated probe reagents linked to two biotinylated nucleic acid labels).

[00178] Biotinylation of a nucleic acid label (i.e., attaching a biotin to a nucleic acid label) can occur at any location of the nucleic acid label. In some embodiments, the nucleic acid label can by synthesized or modified with a terminal biotin, i.e., the nucleic acid label can have a biotin at its 5' end and/or 3' end. In other embodiments, the nucleic acid label can by synthesized or modified with an internal biotin, i.e., the nucleic acid label can have a biotin anywhere between its 5' and 3' ends, but not at its 5' and/or 3' ends. Such internal biotinylation can leave at least

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one end (e.g., both ends) of the nucleic acid label accessible, allowing the nucleic acid label to be circularized after the nucleic acid label has bound, which can in turn, for example, enable rolling circle amplification as described earlier. Chemical modification of the nucleic acid label, e.g., to attach a terminal and/or an internal biotin to the nucleic acid label after synthesis, is within one of skill in the art.

[00179] For some embodiments where the probe reagent is a nucleic acid, an example of using binding pair conjugation is double-stranded nucleic acid conjugation. In this approach, the first part to be linked is conjugated is with linked a first strand first strand of the double-stranded nucleic acid and the second part to be linked is conjugated with the second strand of the double-stranded nucleic acid. Nucleic acids can include, without limitation, defined sequence segments and sequences comprising nucleotides, ribonucleotides, deoxyribonucleotides, nucleotide analogs, modified nucleotides and nucleotides comprising backbone modifications, branchpoints and nonnucleotide residues, groups or bridges.

[00180] In some embodiments, the linker can be a linker molecule. Examples of linker molecules can include, but are not limited to, a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne glycol, crosslinker, or combination thereof.

[00181] Non-limiting examples of crosslinkers that can be used as linker molecules can include, but are not limited to, amine-to-amine crosslinkers (e.g., but are not limited to the ones based on NHS-ester and/or imidoester reactive groups), amine-to-sulfhydryl crosslinkers, carboxyl-to-amine crosslinkers (e.g., but are not limited to, carbodiimide crosslinking agents such as DCC, and/or EDC (EDAC); and/or N-hydroxysuccinimide (NHS)), photoreactive crosslinkers (e.g., but not limited to, aryl azide, diazirine and any art-recognized photo-reactive (light-activated) chemical crosslinking reagents), sulfhydryl-to-carbohydrate crosslinkers (e.g., but are not limited to the ones based on malemide and/or hydrazide reactive groups), sulfhydryl-to hydroxyl crosslinkers (e.g., but are not limited to the ones based on maleimide and/or isocyanate reactive groups), sulfhydryl-to-sulfhydryl crosslinkers (e.g., but are not limited to, maleimide and/or pyridyldithiol reactive groups), sulfo-SMCC crosslinkers, sulfo-SBED biotin label transfer reagents, sulfhydryl-based biotin label transfer reagents, photoreactive amino acids (e.g., but are not limited to diazirine analogs of leucine and/or methionine), NHS-azide Staudinger ligation reagents (e.g., but are not limited to, activated azido compounds), NHS-phosphine Staudinger ligation reagents (e.g., but are not limited to, activated phosphine

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compounds), and any combinations thereof.

[00182] In some embodiments, any commercially available crosslinkers (e.g., but not limited to the ones from Thermo Scientific or Piercenet, Rockford, IL) can be used as a linker molecule herein.

[00183] In some embodiments, the term "linker" as used herein can be a physical substrate. In some embodiments, the physical substrate is a particle. The particle can be of any shape, e.g., spheres; rods; shells; beads, tubes, wires, and prisms; and these particles can be part of a network.

[00184] The particles can be made of any materials. In some embodiments, the particle can comprise a material selected from the group consisting of metal (e.g., gold, or iron), metal oxides (e.g., iron oxide), plastic, glass, polymer (e.g., polystyrene), and any combinations thereof.

[00185] For in vivo purposes, e.g., disease and/or pathogen diagnosis and/or targeted drug delivery, the polymer can be biocompatible and/or biodegradable. The term "biocompatible polymer" refers to polymers which, in the amounts employed, are non-toxic and substantially non-immunogenic when used internally in the patient and which are substantially insoluble in the body fluid of the mammal. Examples of non-biodegradable biocompatible polymers include, by way of example, cellulose acetates (including cellulose diacetate), ethylene vinyl alcohol copolymers, hydrogels (e.g., acrylics), polyacrylonitrile, polyvinylacetate, cellulose acetate butyrate, nitrocellulose, copolymers of urethane/carbonate, copolymers of styrene/maleic acid, and any combinations thereof. Biodegradable polymers are known in the art, e.g., without limitations, linear-chain polymers such as polylactides, polyglycolides, polycaprolactones, polyanhydrides, polyamides, polyurethanes, polyesteramides, polyorthoesters, polydioxanones, polyacetals, polyketals, polycarbonates, polyorthocarbonates, polyphosphazenes, polyhydroxybutyrates, polyhydroxyvalerates, polyalkylene oxalates, polyalkylene succinates, poly(malic acid), poly(amino acids), polyvinylpyrrolidone, polyethylene glycol, polyhydroxycellulose, chitin, chitosa, and copolymers, terpolymers and any combinations thereof. Other biodegradable polymers include, for example, fibrin, gelatin, collagen, and any combinations thereof.

[00186] The particles can be of any size for the purpose of various aspects described herein, provided that the particle size does not significantly affect the diffusion property of the detection reagent. For example, the particle size can range from 5 nm to 1 mm, from about 10 nm to about

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500 μm, or from about 50 nm to about 250 μm. In some embodiments, a particle described herein is a nanoparticle or a microparticle. As used herein, the term "nanoparticle" refers to particles that are on the order of 10^{-9} or one billionth of a meter and below 10^{-6} or 1 millionth of a meter in size. The term "microparticle" as used herein refers to particles that are on the order of 10^{-6} or one millionth of a meter and below 10^{-3} or 1 thousandth of a meter in size. In some embodiments, the particle can be selected from a group consisting of a gold nanoparticle or microparticle, a paramagnetic nanoparticle or microparticle, a magnetic nanoparticle or microparticle, a polystyrene nanoparticle or microparticle, a nanotube or a microtube, a nanowire or a microwire, and any combinations thereof.

[00187] The particles can be adapted to possess at least one additional property, depending on various applications. In some embodiments, the particles can be adapted to be magnetic responsive or paramagnetic-responsive, e.g., magnetic or paramagnetic particles. In some embodiments, the particles can be adapted to be a delivery vehicle. For example, in some embodiments, the particles can be encapsulated with a therapeutic agent, e.g., for targeted drug delivery to treat a disease or disorder.

[00188] In some embodiments, the particles can be modified. For example, the particles can be conjugated with proteins, peptides, nucleic acids, or any combinations thereof. In one embodiment, the particles can be surface-conjugated or coated with one member of the binding pair as described above (e.g., for biotin-streptavidin interaction, streptavidin-coated particles can be used for the purpose of various aspects described herein). In some embodiments, the particles can be surface-activated with functional groups (e.g., but not limited to, amine, carboxylic acid, epoxy, tosyl, silane, and any combinations thereof), e.g., to provide binding sites for probe reagents and/or nucleic acid labels. Methods for surface modifications of particles, such as nanoparticles or microparticles, are well known in the art. One of skill in the art can readily modify or activate the surface of particles using any art-recognized reactions, e.g., covalently through crosslinkers, or through protein interaction.

[00189] The particles described herein can be used in conjunction with the organic linker described above, to form a conjugate linker, or the particles can be used alone as a linker. Both the nucleic acid labels and the probe reagents can be coupled to the particles using a multitude of methods. These include, but are not limited to, direct covalent attachment such as using chemical crosslinkers based on chemistries such as NHS, maleimide, tosyl, isocyanide, etc.; chemical

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linkage such as using EDC chemistry, thiol adsorption to gold, vinyl/acrylate radical reaction, acrylate-based addition (of e.g., thiols); and protein mediated couplings based on proteins such as streptavidin (and its relatives such as avidin, neutravidin, etc.), protein A, and secondary antibodies, and any combinations thereof.

[00190] The particles can act as hubs that facilitate a conjugation of multiple nucleic acid labels to single or multiple probes. Depending on applications and/or properties of nucleic acid labels and/or probe reagents, particles can be used as hubs for multi-conjugation. For example, in some embodiments, by acting as a hub, the particles can allow multiple nucleic acid labels to be present at each location (e.g., location of a target molecule or analyte) where the probe binds. Accordingly, the signal generated from the detection reagent can be amplified and thus greater than if only a single label was present. This is especially desirable where antigens/targets or analytes are sparse in a sample.

[00191] In some embodiments, the particles can allow multiple probes to be arranged in proximity to each other. Thus, the particles can allow several weaker binding events to combine into strong binding. This "avidity action" can transform probe reagents with individually weak affinities into effective sensors.

[00192] In some embodiments, by acting as a hub, the particles can allow each probe reagent to conjugate to multiple nucleic acid labels. This capability can eliminate the need for other amplification methods (e.g., rolling circle amplification) as multiple nucleic acid labels corresponding to a probe reagent can be used as a form of signal amplification. In some embodiments, this capability can also be used to separate the detection reagents with one nucleic acid label from the ones with different nucleic acid labels, which can, in turn, enable, for example, sequencing by single-base extension. Furthermore, different nucleic acid labels can be conjugated to the particles at controlled ratios, and the ratios can encode additional information. By way of example only, to capture in situ information of one or more enzymes' behavior (e.g., relative enzyme concentration) in the presence of an analyte, two or more different nucleic acid labels can be conjugated to the particles, wherein a subpopulation of the nucleic acid labels corresponding to the probe reagent is not cleavable, while other subpopulations of the nucleic acid labels are each adapted to be cleavable in the presence of their respective enzymes. In such embodiments, the cleavable nucleic acid labels can be conjugated to the particles by cleavable peptide bonds specific for corresponding enzymes. By comparing the ratios of signals generated

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from the various nucleic acid labels, one would be able to determine the relative enzyme concentrations in the presence of an analyte.

[00193] The arrangement of the probe reagent(s) and/or nucleic acid label(s) on the particles can vary with a number of factors, e.g., applications, properties of probe reagents and/or nucleic acid labels, sample properties, and/or analytes of interests. In some embodiments, the probe reagents and nucleic acid labels can be conjugated to the particle directly (see, e.g., **Fig. 4A**). In some embodiments, one component can be linked to the particle through the other. In such embodiments, the probe reagents can be conjugated or linked to the particle through the nucleic acid labels (see, e.g., **Fig. 4B**), e.g., to allow the probe reagents being more accessible by the corresponding analytes in a sample.

[00194] In some embodiments, the detection reagents can further comprise at least one substrate linker conjugated to the particle. In such embodiment, the substrate linker can allow the detection reagents to be immobilized to a solid support or substrate.

Detectable molecules or detectable labels

[00195] A detectable molecule or detectable label can be covalently attached to a decoder probe or complementary nucleobase before or after the decoder probe or the complementary nucleobase is attached to the pre-determined subsequences or hybridization sites of a nucleic acid label. For example, in attaching a detectable label to a decoder probe, the label can be covalently attached by incorporation of a nucleotide containing a detectable label into the nucleic acid during its synthesis, but before it is hybridized the pre-determined subsequence of the nucleic acid label. Alternatively, during the synthesis of a decoder probe sequence, a nucleotide containing a detectable label acceptor group can be included, and the detectable label can be added to the decoder probe after its synthesis, either before or after it is hybridized to the pre-determined subsequences of the nucleic acid label. Alternatively, the detectable label can be indirectly attached to the decoder probe, for example, by incorporating a nucleotide containing a ligand-binding molecule (e.g., biotin) into the decoder probe during synthesis, and by adding a ligand (e.g., streptavidin) that is covalently attached to the detectable molecule, or vice versa.

[00196] In some embodiments, the ratios of a detectable label to a decoder probe can range from about 1:1 to about 100:1, from 1:1 to about 50:1, from about 1:1 to about 25:1, from about

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1:1 to about 10:1, or from about 1:1 to about 5:1. When a decoder probe comprises more than one detectable label, each detectable label can be attached to a nucleotide of the decoder probe.

[00197] A detectable label or a detectable molecule can be attached to any nucleotide including both natural and non-natural nucleotides. A nucleotide contains three parts, a phosphate group, a pentose five-carbon sugar molecule, and an organic base. In RNA, the pentose is ribose and in DNA it is deoxyribose and so nucleotides for incorporation into RNA are called ribonucleotides and nucleotides for incorporation into DNA are called deoxyribonucleotides. Three bases adenine, guanine, and cytosine are found in both DNA and RNA while thymine is normally found only in DNA and uracil is normally found only in RNA. Nucleotides can have one, two or three attached phosphate groups and are sometimes referred to as nucleoside phosphates. Nucleotides can contain modified nucleosides having modified bases (e.g., 5-methyl cytosine) and modified sugar groups (e.g., 2′O-methyl ribosyl, 2′O-methoxyethyl ribosyl, 2′fluoro ribosyl, 2′amino ribosyl, and the like). An example of non-natural bases that are used in the art are isocytidine and isoguanine.

[00198] A detectable label or a detectable molecule as used herein is intended to mean an individual measurable moiety, such as a radioisotope, fluorochrome, dye, enzyme (including its effect on a substrate), nanoparticle, chemiluminescent marker, biotin, or other moiety known in the art that is measurable by analytical methods. A detectable label or a detectable molecule can be attached to a nucleotide using methods well known in the art and exemplified herein.

[00199] Without limitations, examples of a detectable label or a detectable molecule that can be utilized by some aspects described herein can include optical reporters or optical labels. Suitable optical reporters or optical labels include, but are not limited to, fluorescent reporters and chemiluminescent groups. A wide variety of fluorescent reporter dyes are known in the art. Typically, the fluorophore is an aromatic or heteroaromatic compound and can be a pyrene, anthracene, naphthalene, acridine, stilbene, indole, benzindole, oxazole, thiazole, benzothiazole, cyanine, carbocyanine, salicylate, anthranilate, coumarin, fluorescein, rhodamine or other like compound. Suitable fluorescent reporters include xanthene dyes, such as fluorescein or rhodamine dyes, including, but not limited to, Alexa Fluor® dyes (InvitrogenCorp.; Carlsbad, Calif), fluorescein, fluorescein isothiocyanate (FITC), Oregon GreenTM, rhodamine, Texas red, tetrarhodamine isothiocynate (TRITC), 5-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G),

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N,N,N,N'-tetramefhyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX). Suitable fluorescent reporters also include the naphthylamine dyes that have an amino group in the alpha or beta position. For example, naphthylamino compounds include l-dimethylamino-naphthyl-5-sulfonate, l-anilino-8-naphthalene sulfonate, 2-p-toluidinyl-6-naphthalene sulfonate, and 5-(2'-aminoethyl)aminonaphthalene-l-sulfonic acid (EDANS). Other fluorescent reporter dyes include coumarins, such as 3-phenyl-7-isocyanatocoumarin; acridines, such as 9-isothiocyanatoacridine and acridine orange; N-(p(2-benzoxazolyl)phenyl)maleimide; cyanines, such as Cy2, indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-(-carboxy-pentyl)-3'ethyl-5,5'-dimethyloxacarbocyanine (CyA); 1H,5H,11H, 15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-l-pyrrolidinyl)oxy]-6-oxohexyl] amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17octahydro-inner salt (TR or Texas Red); BODIPYTM dyes; benzoxadiazoles; stilbenes; pyrenes; and the like. Many suitable forms of these fluorescent compounds are available and can be used.

[00200] Examples of fluorescent proteins suitable for use as imaging agents include, but are not limited to, green fluorescent protein, red fluorescent protein (e.g., DsRed), yellow fluorescent protein, cyan fluorescent protein, blue fluorescent protein, and variants thereof (see, e.g., U.S. Pat. Nos. 6,403, 374, 6,800,733, and 7,157,566). Specific examples of GFP variants include, but are not limited to, enhanced GFP (EGFP), destabilized EGFP, the GFP variants described in Doan et al, *Mol. Microbiol,* 55:1767-1781 (2005), the GFP variant described in Crameri et al, *Nat. Biotechnol.,* 14:315319 (1996), the cerulean fluorescent proteins described in Rizzo et al, *Nat. Biotechnol,* 22:445 (2004) and Tsien, *Annu. Rev. Biochem.,* 67:509 (1998), and the yellow fluorescent protein described in Nagal et al, *Nat. Biotechnol.,* 20:87-90 (2002). DsRed variants are described in, e.g., Shaner et al, *Nat. Biotechnol.,* 22:1567-1572 (2004), and include mStrawberry, mCherry, morange, mBanana, mHoneydew, and mTangerine. Additional DsRed variants are described in, e.g., Wang et al, *Proc. Natl. Acad. Sci. U.S.A.,* 101:16745-16749 (2004) and include mRaspberry and mPlum. Further examples of DsRed variants include mRFPmars described in Fischer et al, *FEBS Lett.,* 577:227-232 (2004) and mRFPruby described in Fischer et al, *FEBS Lett,* 580:2495-2502 (2006).

[00201] Amine-reactive and thiol-reactive fluorophores are available and generally used for labeling nucleotides and biomolecules. In some embodiments, nucleotides are fluorescently labeled during chemical synthesis, for example, incorporation of amines or thiols during

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nucleotide synthesis permit addition of fluorophores. Fluorescently labeled nucleotides are commercially available. For example, uridine and deoxyuridine triphosphates are available that are conjugated to ten different fluorophores that cover the spectrum.

[00202] In some embodiments, radioisotopes can be utilized as detectable molecules or detectable molecules for labeling nucleotides including, for example, ^{32}P, ^{33}P, ^{35}S, ^{3}H, and ^{125}I. These radioisotopes have different half-lives, types of decay, and levels of energy which can be tailored to match the needs of a particular experiment. For example, ^{3}H is a low energy emitter which results in low background levels, however this low energy also results in long time periods for autoradiography. Radioactively labeled ribonucleotides and deoxyribonucleotides are commercially available. Nucleotides are available that are radioactively labeled at the first, or α, phosphate group, or the third, or γ, phosphate group. For example, both [α-^{32}P]dATP and [γ-^{32}P]dATP are commercially available. In addition, different specific activities for radioactively labeled nucleotides are also available commercially and can be tailored for different experiments.

[00203] Suitable non-metallic isotopes include, but are not limited to, ^{11}C, ^{14}C, ^{13}N, ^{18}F, ^{123}I, ^{124}I, and ^{125}I. Suitable radioisotopes include, but are not limited to, ^{99}mTc, ^{95}Tc, ^{111}In, ^{62}Cu, ^{64}Cu, Ga, ^{68}Ga, and ^{153}Gd. Suitable paramagnetic metal ions include, but are not limited to, Gd(III), Dy(III), Fe(III), and Mn(II). Suitable X-ray absorbers include, but are not limited to, Re, Sm, Ho, Lu, Pm, Y, Bi, Pd, Gd, La, Au, Au, Yb, Dy, Cu, Rh, Ag, and Ir. In some embodiments, the radionuclide is bound to a chelating agent or chelating agent-linker attached to the aggregate. Suitable radionuclides for direct conjugation include, without limitation, ^{18}F, ^{124}I, ^{125}I, ^{131}I, and mixtures thereof. Suitable radionuclides for use with a chelating agent include, without limitation, ^{47}Sc, ^{64}Cu, ^{67}Cu, ^{89}Sr, ^{86}Y, ^{87}Y, ^{90}Y, ^{105}Rh, ^{111}Ag, ^{111}In, ^{117}mSn, ^{149}Pm, ^{153}Sm, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, ^{211}At, ^{212}Bi, and mixtures thereof. Suitable chelating agents include, but are not limited to, DOTA, BAD, TETA, DTPA, EDTA, NTA, HDTA, their phosphonate analogs, and mixtures thereof. One of skill in the art will be familiar with methods for attaching radionuclides, chelating agents, and chelating agent-linkers to the particles.

[00204] Non-radioactive and non-fluorescent label monomers are also available. For example, biotin can be attached directly to nucleotides and detected by specific and high affinity binding to avidin or streptavidin which has been chemically coupled to an enzyme catalyzing a colorimetric reaction (such as phosphatase, luciferase, or peroxidase). Digoxigenin labeled

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nucleotides can also similarly be used for non-isotopic detection of nucleic acids. Biotinylated and digoxigenin-labeled nucleotides are commercially available.

[00205] In some embodiments, enzymatic reaction on a substrate can be utilized a detection method. In such embodiments, enzymes (e.g., horseradish peroxidase or alkaline phosphatase) can be linked to a nucleotide of a nucleic acid label and/or decoder probe. During detection, a substrate on which the particular enzyme reacts can be added to induce a colorimetric reaction. Any enzyme-substrate reactions known in the art can be used herein.

[00206] Very small particles, termed nanoparticles, also can be used as detectable labels or detectable molecules to label decoder probes or any nucleic acids. These particles range from 1-1000 nm in size and include diverse chemical structures such as gold and silver particles and quantum dots.

[00207] When irradiated with angled incident white light, silver or gold nanoparticles ranging from 40-120 nm will scatter monochromatic light with high intensity. The wavelength of the scattered light is dependent on the size of the particle. Four to five different particles in close proximity will each scatter monochromatic light which when superimposed will give a specific, unique color. Alternatively, the gold nanoparticles can be detected or "developed" using a silver-based developer such that the tiny nanoparticle can be detected easily, even with naked eyes. The particles are being manufactured by companies such as Genicon Sciences. Derivatized silver or gold particles can be generally attached to a broad array of molecular molecules including, proteins, antibodies, small molecules, receptor ligands, and nucleic acids. For example, the surface of the particle can be chemically derivatized to allow attachment to a nucleotide, which can then be incorporated into a decoder probe.

[00208] Another type of nanoparticle that can be used as a detectable molecule or detectable label are quantum dots. Quantum dots are fluorescing crystals 1-5 nm in diameter that are excitable by a large range of wavelengths of light. These crystals emit light, such as monochromatic light, with a wavelength dependent on their chemical composition and size. Quantum dots such as CdSe, ZnSe, InP, or InAs possess unique optical properties.

[00209] Due to their very small size the quantum dots can be generally coupled into oligonucleotides directly without affecting the solubility or use of the oligonucleotide. Thus, quantum dots can be coupled to decoder probes as described herein. To synthesize a decoder probe-quantum dot complex by conventional batch chemistry, both the decoder probe and the

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quantum dot require at least a reactive group of different kinds that can be reacted with each other. For example, if a decoder probe has an amino group and a quantum dot has an aldehyde group, these groups can react to form a Schiff base. A decoder probe can be derivatized to attach a single amino or other functional group using chemistry well known in the art. When a quantum dot is derivatized, the quantum dot can be covered with a chemical reagent which results in coating the entire surface of the nanoparticle with several functional groups.

[00210] A detectable molecule or detectable label can be attached to a nucleotide of a decoder probe or nucleic acid using a variety of methods well known in the art and described herein. For example, the detectable molecule or detectable label can be directly attached to the nucleotide in a 1:1 correspondence by incorporation of a radioactive phosphate into the phosphate backbone of the nucleotide. Also, for example, a general method for labeling phosphates with a fluorescent label that employs an imidazole derivative prepared from a BODIPY FL hydrazide has been reported (Wang and Giese, Anal. Chem. 65: 3518 (1993).

[00211] Depending on the labeling moiety used, it can be desirable to derivatize or chemically modify a nucleotide in order to bind the label monomer. These methods and chemistries are known in the art. In addition, a linker can be used to attach a detectable molecule or detectable label to a nucleotide in a 1:1 correspondence. For example, a fluorescently labeled nucleotide such as fluorescein-12-dUTP can have a fluorophore monomer attached via a four-atom aminoalkynyl group to the dUTP molecule.

[00212] These nucleotides attached to detectable molecules or detectable labels can be incorporated into a decoder probe or nucleic acid using several methods for labeling nucleic acids well known in the art. For example, enzymes such as DNA or RNA polymerases, Taq polymerases, terminal deoxynucleotidyl transferases, or reverse transcriptases can be used to incorporate labeled nucleotides into decoder probes or nucleic acids.

[00213] Labeled nucleotides can be incorporated into decoder probe sequences or nucleic acids, for example, by nick translation. In this procedure DNAse I is used to create single-strand nicks in double stranded DNA and then the 5′ to 3′ exonuclease and 5′ to 3′ polymerase actions of E. coli DNA polymerase I are used to remove stretches of single stranded DNA starting at the nicks and replace them with new strands made by incorporation of labeled nucleotides. Nick translation can utilize any labeled nucleotide including radioactively labeled nucleotides and biotinylated or digoxigenin labeled nucleotides. In a similar way T4 DNA polymerase can be

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used to incorporate labeled nucleotides. In addition, labeled nucleotides can be incorporated into nucleic acids using the polymerase chain reaction (PCR) and Taq polymerases. The degree of labeling can be controlled by including one, or up to all four labeled nucleotides. In addition, the degree of labeling can be controlled by increasing or decreasing the concentration of the labeled nucleotide(s).

[00214] Other methods for labeling decoder probes or nucleic acids include generating single-stranded cDNA from RNA by using a reverse transcriptase in the presence of labeled nucleotides. In addition, DNA can be cloned into a vector with SP6 or T7 polymerase sites. Transcription in the presence of SP6 or T7 RNA polymerase and labeled nucleotides results in a labeled RNA transcript. The transcript can be labeled to different degrees by including one or more labeled nucleotides. In addition, several nucleotides within a nucleic acid can be labeled, for example, by cloning DNA into a bacteriophage M13 based vector. Then the Klenow fragment of DNA polymerase I and the M13 universal probe primer can be used to synthesize the complementary stand with incorporation of labeled nucleotides.

[00215] Several methods are described above for incorporation of labeled nucleotides into newly synthesized decoder probes or nucleic acids. Existing nucleic acids can also be labeled using several methods known in the art. For example, RNA or DNA can be end-labeled with [γ-32P]ATP and T4 polynucleotide kinase. This kinase can be used to transfer the radioactive phosphate of ATP to a free 5′ OH group in either DNA or RNA. The enzyme also has a phosphatase activity and so two reactions are possible. In the forward reaction, the enzyme catalyzes phosphorylation following removal of 5′ terminal phosphates with alkaline phosphatase (or other phosphatase). In the exchange reaction, the kinase catalyzes the exchange of an existing 5′ phosphate with the third or γ phosphate of ATP. The latter reaction is carried out in the presence of excess ATP and ADP for efficient phosphorylation. Using this method the radioactive phosphate of ATP is transferred to the end of the nucleic acid molecule.

[00216] Decoder probes or nucleic acids can also be labeled with terminal deoxynucleotidyl transferase which adds labeled nucleotides onto the 3′ end of DNA fragments. Both single and double-stranded DNAs are substrates for this enzyme. The large (Klenow) fragment of E. coli DNA polymerase I can also be used to label the ends of decoder probes or nucleic acids. Since this enzyme has a 5′ to 3′ polymerase activity it can be used to "fill in" the 3′ ends of nucleic acid (e.g., DNA) fragments opposite of 5′ extensions or overhangs with labeled nucleotides. End-

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labeling of decoder probes or nucleic acids using polynucleotide kinase or terminal deoxynucleotidyl transferase results in the incorporation of one detectable label or detectable molecule per nucleic acid. The "fill in" reaction can be used to label the decoder probe or nucleic acid at one nucleotide per nucleic acid or at more than one nucleotide per nucleic acid.

[00217] In addition, decoder probes or nucleic acids can be labeled by modification of nucleotides within the nucleic acid sequences. For example, cytidine residues in DNA and RNA can be modified by reaction with sodium bisulfite to form sulfonate intermediates that can then be directly coupled to hydrazides or aliphatic amines. Virtually any of the fluorescent, biotin or other hydrazides or aliphatic amines can be used in this reaction. The bisulfite-activated cytidylic acid can also be coupled to aliphatic diamines such as ethylenediamine. The amine-modified nucleic acids (e.g., DNA or RNA) can then be modified with any of the amine-reactive dyes. In addition, phosphate groups can be targeted in nucleic acids for labeling. Although phosphate groups of nucleotides are not very reactive in aqueous solution, their terminal phosphate groups can react with carbodiimides and similar reagents in combination with nucleophiles to yield labeled phsophodiesters, phosphoramidates and phosphorothioates. For example, nucleic acids (e.g., DNA) can be reacted quantitatively with carbonyl diimidazole and a diamine such as ethylenediamine to yield a phosphoramidate that has a free primary amine and that this amine can then be modified with amino-reactive reagents. Fluorescent or biotinylated amines have been coupled to the 5′ phosphate of tRNA using dithiodipyridine and triphenylphosphine.

[00218] The bond between detectable molecules or detectable labels and decoder probes or nucleic acids can be covalent bonds or non-covalent bonds that are stable to hybridization. The detectable molecules or detectable labels can be bound to a decoder probe or a nucleic acid in a sequence specific manner, for example by the incorporation of a labeled nucleotide into a sequence that has been digested by a restriction enzyme. Alternatively the detectable molecules or detectable labels can be bound to a decoder probe or a nucleic acid in a non-sequence specific manner, for example by the incorporation of a label onto the terminal phosphate of a nucleic acid using [γ-^{32}P]ATP and T4 polynucleotide kinase.

Synthesis of nucleic acids (e.g., for at least part of the nucleic acid label such as pre-determined subsequences and/or decoder probe sequences

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[00219] The nucleic acids described herein can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the label attachment region and the annealed complementary polynucleotide sequences or segments, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the synthetic nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5′-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine.

[00220] Alternatively, the synthetic nucleic acid can be produced biologically using a vector into which a nucleic acid has been subcloned. As one example, a linear single-stranded DNA backbone can be made from a double stranded plasmid DNA using a four step protocol that includes (i) linearization of the dsDNA with a restriction enzyme, (ii) dephosphorylation with a thermolabile phosphatase, (iii) digestion with a second restriction enzyme to separate the cloning vector from the backbone sequence, and (iv) digestion with a strand-specific lambda exonuclease digestion, leaving only one strand of the backbone fragment intact.

[00221] In various embodiments, the nucleic acids can be modified at the base moiety, sugar moiety or phosphate backbone to improve, e.g., the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup et al, 1996, Bioorganic & Medicinal Chemistry 40:5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, e.g., DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral

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backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup et al, 1996, Bioorganic & Medicinal Chemistry 4(1): 5-23; Perry-O'Keefe et al., 1996, Proc. Natl. Acad. Sci. USA 93: 14670-675.

[00222] In an exemplary embodiment, the selected novel nucleic acid sequence (e.g., DNA sequence) can be constructed synthetically as double-stranded nucleic acid by a commercial gene synthesis company and cloned in an oriented fashion into a "phagemid", a plasmid vector containing an M13 or f1 phage intergenic (IG) region which contains the cis-acting sequences necessary for nucleic acid (e.g., DNA) replication and phage encapsidation, such as pUC119. The appropriate orientation of the cloned insert relative to the phage origin of replication allows for the generation of a single-stranded DNA backbone which is the reverse complement of the RNA molecules generated by in vitro transcription for each label attachment region.

[00223] In order to generate the single-stranded nucleic acid (e.g., DNA) backbone (e.g., of at least part of the nucleic acid label and/or decoder probes), the phagemid is transformed into an E. coli strain containing an F′ episome. Subsequent infection of the transformed bacteria with a helper phage such as the M113 mutant K07 results in the secretion of the phagemid carrying the single-stranded nucleic acid sequence, packaged phage from which the circular, single-stranded nucleic acid is prepared using a standard protocol. This nucleic acid is linearized and the vector portion is excised by annealing short, complementary oligonucleotides to either end of the single-strander nucleic acid sequence to generate double-stranded restriction sites, followed by treatment with the appropriate restriction enzymes.

Analytes or target molecules

[00224] The terms "analyte," "target analyte," and "target molecule", as used interchangeably herein, refer to the molecule detected, identified or measured by binding of a detection reagent described herein whose probe reagent(s) recognize (i.e., are specific binding partners) thereto. In some embodiments, a target molecule or an analyte can be, but is not limited to, any of the following or any combinations of the following: nucleic acid, peptide, a polypeptide/protein (e.g., a bacterial or viral protein or an antibody), a lipid, a carbohydrate, a glycoprotein, a glycolipid, a small molecule, an organic monomer, sugar, peptidoglycan, a cell, a virus or a drug.

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Nucleic acids that can be analyzed by the methods herein include: double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA (e.g. mRNA or miRNA) and RNA hairpins. Generally, a target molecule can be a naturally occurring molecule or a cDNA of a naturally occurring molecule or the complement of said cDNA. In other embodiments, a target molecule can be modified, e.g., by mutation or chemical reaction. In some embodiments, a target molecule can be synthetic or recombinant.

[00225] In some embodiments, an analyte can be an analyte comprising at least one post-translational modification, e.g., phosphorylations and/or glycosylations.

[00226] A target molecule or an analyte can be part of a sample that contains other components or can be the sole or major component of the sample. A target molecule or an analyte can be a component of a whole cell, tissue or body fluid, a cell or tissue extract, a fractionated lysate thereof or a substantially purified molecule. The target molecule can be present in solution or attached to a solid substrate, including, for example, to a solid surface such as a chip, microarray, bead or a blotting membrane. Also the target molecule or analyte can have either a known or unknown structure or sequence.

Sample

[00227] The methods, detection reagents and kits described herein can be used to analyze a sample from any sources, e.g., but not limited to biological samples (e.g., collected from organisms, animals or subjects), environmental samples, food, food byproduct, soil, archaeological samples, extraterrestrial samples, or any combinations thereof.

[00228] In some embodiments, the term "sample" refers to a biological sample. The term "biological sample" as used herein denotes a sample taken or isolated from a biological organism, e.g., tissue cell culture supernatant, cell lysate, a tissue sample (e.g., biopsy), a homogenate of a tissue sample from a subject or a fluid sample from a subject. Exemplary biological samples include, but are not limited to, blood, sputum, urine, cerebrospinal fluid, urine, sweat, mucus, nasal discharge, vaginal fluids, spinal fluid, pleural fluid, nipple aspirates, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, feces, sperm, cells or cell cultures, serum, leukocyte fractions, smears, tissue samples of all kinds, plants and parts of plants, microorganisms (such as bacteria), viruses (such as cytomegalo virus, HIV, hepatitis B, hepatitis C, hepatitis δ virus), yeasts, embryos, fungi, cell-

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free sample material, etc. The term also includes both a mixture of the above-mentioned samples such as fungus-infected plants or whole human blood containing mycobacteria as well as food samples that contain free or bound nucleic acids, or proteins, or cells containing nucleic acids or proteins, environmental samples which contain free or bound nucleic acids, or proteins, or cells containing nucleic acids or proteins. The term "biological sample" also includes untreated or pretreated (or pre-processed) biological samples.

[00229] A "biological sample" can contain cells from subject, but the term can also refer to non-cellular biological material, such as non-cellular fractions of blood, saliva, or urine, that can be used to measure gene expression levels. In some embodiments, the sample is from a resection, bronchoscopic biopsy, or core needle biopsy of a primary or metastatic tumor, or a cell block from pleural fluid. In addition, fine needle aspirate samples can be used. Samples can be either paraffin-embedded or frozen tissue. In some embodiments, a biological sample can comprise a biopsy, a surgically removed tissue, a swap, or any combinations thereof.

[00230] The sample can be obtained by removing a sample of cells from a subject, but can also be accomplished by using previously isolated cells (e.g. isolated by another person). In addition, the biological sample can be freshly collected or a previously collected sample. Furthermore, the biological sample can be utilized for the detection of the presence and/or quantitative level of a biomolecule of interest. Representative target analytes include, but are not limited to nucleic acids, proteins, and derivatives and fragments thereof.

[00231] In some embodiments, the biological sample is an untreated biological sample. As used herein, the phrase "untreated biological sample" refers to a biological sample that has not had any prior sample pre-treatment except for dilution and/or suspension in a solution. Exemplary methods for treating a biological sample include, but are not limited to, centrifugation, filtration, sonication, homogenization, heating, freezing and thawing, and any combinations thereof.

[00232] In accordance with some embodiments of various aspects described herein, a biological sample can be pre-processed, as described earlier, before employing the detection reagents and the methods described herein. In some embodiments, the biological sample can be filtered before isolating a cellular material according to the methods, apparatus and kits described herein.

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[00233] In some embodiments, the biological sample can be treated with a chemical and/or biological reagent. Chemical and/or biological reagents can be employed to protect and/or maintain the stability of the sample or target analytes during processing. In addition, or alternatively, chemical and/or biological reagents can be employed to release or expose target analytes from other components of the sample. One exemplary reagent is a protease inhibitor, which is generally used to protect or maintain the stability of the target analytes during processing.

[00234] In some embodiments, the term "sample" as used herein can refer to an environmental sample (including, but not limited to, air, agricultural (e.g., but not limited to hydrofarms or hydroponic samples), pond, water, wastewater, and soil samples); biological warfare agent samples; research samples including extracellular fluids. In some embodiments, an environmental sample can comprise a sample collected from a working surface of an equipment or machine (e.g., but not limited to, food or pharmaceutical product processing equipment or machine), a device (e.g., but not limited to, biomedical devices, implantation devices, fluid delivery devices such as a tubing, and/or a catheter), and/or a building or dwellings (e.g., but not limited to, food processing plants, pharmaceutical manufacturing plants, hospitals, and/or clinics).

[00235] In some embodiments, a sample can comprise food (e.g., solid and/or fluid food as well as processed food) and/or food byproduct. For example, the methods, detection reagents and kits described herein can be used to detect an analyte, e.g., a particular nutrient, in food and/or food byproduct, e.g., but not limited to, meat, milk, yoghurt, bread, starch-based products, vegetables, and any combinations thereof. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in food and/or food byproduct.

[00236] In some embodiments, a sample can comprise a pharmaceutical product (e.g., but not limited to pills, tablets, gel capsules, syrups, vaccines, liquids, sprays, and any combinations thereof). For example, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of a particular active agent present in a pharmaceutical product. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in a pharmaceutical product.

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[00237] In some embodiments, a sample can comprise an archaeological sample. In some embodiments, an archaeological sample can be obtained or collected from artifacts, architecture, biofacts (or ecofact, e.g., an object found at an archaeological site), cultural landscapes, and any combinations thereof.

[00238] In some embodiments, a sample can comprise an extraterrestrial sample. For example, an extraterrestrial sample can be any object or specimen (e.g., rock, meteorite, and/or environmental samples) obtained or collected from outer space or universe, and/or planets beyond the planet Earth, e.g., the moon, other planets (e.g., but not limited to, Mars, and/or Jupiter) and/or non-stellar objects.

Applications of the detection reagents

[00239] The detection reagents, compositions, methods and kits described herein can be used for diagnostic, prognostic therapeutic and screening purposes. The inventions described herein provide the advantage that many different target analytes can be analyzed at one time from a single sample using the methods described herein. This allows, for example, for several diagnostic tests to be performed on one sample.

[00240] When the probe reagent is a nucleic acid, the methods described herein can discriminate between nucleotide sequences. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected. In some embodiments, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions. As a result, the process described herein is able to detect various kinds of diseases or disorders, e.g., but not limited to, infectious diseases, genetic diseases, and cancer.

[00241] Without wishing to be bound, the detection reagents, compositions, methods and kits described herein can also be used for environmental monitoring, forensics, and food science. Examples of genetic analyses that can be performed on nucleic acids include e.g., SNP detection, STR detection, RNA expression analysis, promoter methylation, gene expression, virus detection, viral subtyping and drug resistance.

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[00242] In the area of environmental monitoring, some embodiments of various aspects described herein can be used for detection, identification, and monitoring of pathogenic and indigenous microorganisms in natural and engineered ecosystems and microcosms such as in municipal waste water purification systems and water reservoirs or in polluted areas undergoing bioremediation. It can also be used to detect plasmids containing genes that can metabolize xenobiotics, to monitor specific target microorganisms in population dynamic studies, or either to detect, identify, or monitor genetically modified microorganisms in the environment and in industrial plants.

[00243] Some embodiments of various aspects described herein can also be used in a variety of forensic areas, including for human identification for military personnel and criminal investigation, paternity testing and family relation analysis, HLA compatibility typing, and screening blood, sperm, or transplantation organs for contamination.

[00244] In the food and feed industry, some embodiments of various aspects described herein have a wide variety of applications. For example, it can be used for identification and characterization of production organisms such as yeast for production of beer, wine, cheese, yogurt, bread, etc. Another area of use is related to the quality control and certification of products and processes (e.g., livestock, pasteurization, and meat processing) for contaminants. Other uses include the characterization of plants, bulbs, and seeds for breeding purposes, identification of the presence of plant-specific pathogens, and detection and identification of veterinary infections.

[00245] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of a particular active agent present in a pharmaceutical product. In some embodiments, the methods, detection reagents and kits described herein can be used to detect a contaminant, e.g., bacteria, fungus, spores, molds, and/or viruses, in a pharmaceutical product.

[00246] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of an analyte of interest present in an archaeological sample, e.g., obtained or collected from artifacts, architecture, biofacts (or ecofact, e.g., an object found at an archaeological site), cultural landscapes, and any combinations thereof.

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[00247] In some embodiments, the methods, detection reagents and kits described herein can be used to detect the presence and/or measure the level of an analyte of interest present in an extraterrestrial sample, e.g., any object or specimen (e.g., rock, meteorite, and/or environmental samples) obtained or collected from outer space or universe, and/or planets beyond the planet Earth, e.g., the moon, other planets (e.g., but not limited to, Mars, and/or Jupiter) and/or non-stellar objects. For example, the methods, detection reagents, and kits described herein can be used to analyze the composition of an extratrerresterial sample or specimen, e.g., a rock specimen, a water sample, and/or an air sample.

[00248] In some embodiments, the detection reagents and methods described herein can be used to detect and/or identify analytes comprising a post-translation modification, e.g., phosphorylation or glycosylation. As such, in some embodiments, the detection reagents and methods described herein can differentiate between multiple different glycosylation forms of recombinant therapeutic proteins

[00249] ***Immunohistochemistry***: Some embodiments of various aspects described herein can be used to perform antibody-based staining of cell or tissues for microscopic evaluation. This can involve either unfixed or unpermeabilized samples examined for surface or extracellular antigens, or permeabilized samples wherein intracellular antigens are also probed. While very common, immunohistochemistry is typically limited to probing with a small set of antibodies at a time (due to the limitation of available optical colors), and multiple staining cycles are generally avoided, since the stripping of the preceding cycle's antibodies can damage the sample. Some embodiments of the detection reagents comprising antibodies as probe reagents and nucleic acid labels can allow for many antibodies to be used concurrently, which saves time and sample material, extract more data, and demand less prior knowledge of the sample. In some embodiments, without wishing to be bound by theory, when a subset of the probed antigens can overlap spatially, the detection reagents with a plurality of contiguous pre-determined subsequences (e.g., each pre-determined subsequences contain one nucleotide, as shown in **Fig. 1**) can be used.

[00250] ***In-situ hybridization:*** Fluorescence in-situ hybridization (FISH) is a technique for detecting (and/or quantifying) the presence of certain cellular DNA or RNA (often ribosomal RNA). As with other fluorescence-based techniques, FISH is limited to the number of colors available to the microscopy. Using some embodiments of the detection reagents and/or methods

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described herein, the cells can be probed for many sequences simultaneously, thus allowing the user to save time and sample material, extract more data, and demand less prior knowledge of the sample.

[00251] ***Expression profiling***: Nucleic-acid probe reagents of the detection reagents can target messenger RNA or micro RNA and provide information on the RNA-level expression state of the cell. Using some embodiments of the detection reagents and/or methods described herein, the assay can capture and probe numerous mRNA and/or miRNA at once, reducing and/or eliminating potentially harmful stripping steps.

[00252] ***Western blots:*** Western blots are protein assays wherein proteins are separated electrophoretically, transferred to a membrane and stained. In many cases, the staining is done using one or a few antibodies in order to detect the corresponding antigens in the blot. By using some embodiments of the detection reagents and/or methods described herein, the blot can be simultaneously probed using a large number of antibodies without antibody-stripping steps. This can allow one blot to provide significantly more information than the conventional Western blots where one antibody is usually probed at a time and can require antibody stripping for the next antibody probing, thereby saving sample material and time.

[00253] ***Diagnostic/Prognostic Methods:*** The present methods can be applied to the analysis of biological samples obtained or derived from a patient so as to determine whether a diseased cell type is present in the sample and/or to stage the disease.

[00254] In some embodiments, the methods described herein are used in the diagnosis of a condition. As used herein the term "diagnose" or "diagnosis" of a condition includes predicting or diagnosing the condition, determining predisposition to the condition, monitoring treatment of the condition, diagnosing a therapeutic response of the disease, and prognosis of the condition, condition progression, and response to particular treatment of the condition. For example, a blood sample can be assayed according to any of the methods described herein to determine the presence and/or quantity of markers of a cancerous cell type in the sample, thereby diagnosing or staging the cancer.

[00255] In some embodiments, the detection reagents and methods described herein can be directly used as contrast agents for in vivo or in situ diagnosis, in which detection reagents are administered to a subject, either by oral administration or local injection. The probe reagents of the detection reagents can bind to the target analytes, e.g., biomarkers for specific diseases or

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disorders, and detection of the nucleic acid labels using the methods described herein can then locate the target analytes. In some embodiments, the detection reagents and methods described herein can be used to locate microscopic tumors in a subject, where other conventional methods are not sensitive enough to detect such microscopic tumors.

[00256] Cancers which can be detected by some embodiments of the process described herein generally involve oncogenes, tumor suppressor genes, or genes involved in DNA amplification, replication, recombination, or repair. Examples of these include: BRCA1 gene, p53 gene, APC gene, Her2/Neu amplification, Bcr/Ab1, K-ras gene, and human papillomavirus Types 16 and 18. Some embodiments of various aspects can be used to identify amplifications, large deletions as well as point mutations and small deletions/insertions of the above genes in the following common human cancers: leukemia, colon cancer, breast cancer, lung cancer, prostate cancer, brain tumors, central nervous system tumors, bladder tumors, melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and ovarian carcinomas, head and neck tumors, and cervical neoplasms.

[00257] Genetic diseases can also be detected by some embodiments of the process described herein. This can be carried out by prenatal or post-natal screening for chromosomal and genetic aberrations or for genetic diseases. Examples of detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis, Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down Syndrome or other trisomies, heart disease, single gene diseases, HLA typing, phenylketonuria, sickle cell anemia, Tay-Sachs Disease, thalassemia, Klinefelter Syndrome, Huntington Disease, autoimmune diseases, lipidosis, obesity defects, hemophilia, inborn errors of metabolism, and diabetes.

[00258] Alternatively, the methods described herein can be used to diagnose pathogen infections, for example infections by intracellular bacteria and viruses, by determining the presence and/or quantity of markers of bacterium or virus, respectively, in the sample.

[00259] A wide variety of infectious diseases can be detected by some embodiments of the process described herein. Typically, these are caused by bacterial, viral, parasite, and fungal infectious agents. The resistance of various infectious agents to drugs can also be determined using some embodiments of various aspects described herein.

[00260] Bacterial infectious agents which can be detected by some embodiments of various aspects described herein can include Escherichia coli, Salmonella, Shigella, Klebsiella,

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Pseudomonas, Listeria monocytogenes, Mycobacterium tuberculosis, Mycobacterium aviumintracellulare, Yersinia, Francisella, Pasteurella, Brucella, Clostridia, Bordetella pertussis, Bacteroides, Staphylococcus aureus, Streptococcus pneumonia, B-Hemolytic strep., Corynebacteria, Legionella, Mycoplasma, Ureaplasma, Chlamydia, Neisseria gonorrhea, Neisseria meningitides, Hemophilus influenza, Enterococcus faecalis, Proteus vulgaris, Proteus mirabilis, Helicobacter pylori, Treponema palladium, Borrelia burgdorferi, Borrelia recurrentis, Rickettsial pathogens, Nocardia, and Acitnomycetes.

[00261] Fungal infectious agents which can be detected by some embodiments of various aspects described herein can include Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Candida albicans, Aspergillus fumigautus, Phycomycetes (Rhizopus), Sporothrix schenckii, Chromomycosis, and Maduromycosis.

[00262] Viral infectious agents which can be detected by some embodiments of various aspects described herein can include human immunodeficiency virus, human T-cell lymphocytotrophic virus, hepatitis viruses (e.g., Hepatitis B Virus and Hepatitis C Virus), Epstein-Barr Virus, cytomegalovirus, human papillomaviruses, orthomyxo viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses, polio viruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo viruses.

[00263] Parasitic agents which can be detected by some embodiments of various aspects described herein can include Plasmodium falciparum, Plasmodium malaria, Plasmodium vivax, Plasmodium ovale, Onchoverva volvulus, Leishmania, Trypanosoma spp., Schistosoma spp., Entamoeba histolytica, Cryptosporidum, Giardia spp., Trichimonas spp., Balatidium coli, Wuchereria bancrofti, Toxoplasma spp., Enterobius vermicularis, Ascaris lumbricoides, Trichuris trichiura, Dracunculus medinesis, trematodes, Diphyllobothrium latum, Taenia spp., Pneumocystis carinii, and Necator americanis.

[00264] In some embodiments, contacting a biological sample with the detection reagents described herein immune-staining, in-situ hybridization or a combination, can be used for the purpose of detecting and identifying pathogens in biological samples. In such embodiments, for example, surface or intracellular antigens, DNA, ribosomal RNA and/or messenger RNA can be detected with some embodiments of the detection reagents and methods described herein. Detection reagents and methods described herein can be especially useful in this context since a)

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which pathogen may be in the sample is not known in advance, and b) each pathogen cell should respond to one or few of the probes, facilitating the detection reagent and nucleic acid label design.

[00265] Some embodiments of various aspects described herein can also be used for detection of drug resistance by infectious agents. For example, vancomycin-resistant Enterococcus faecium, methicillin-resistant Staphylococcus aureus, penicillin-resistant Streptococcus pneumoniae, multi-drug resistant Mycobacterium tuberculosis, and AZT-resistant human immunodeficiency virus can all be identified with some embodiments of various aspects described herein.

[00266] Thus, the target molecules detected using the compositions and methods described herein can be either patient markers (such as a cancer marker) or markers of infection with a foreign agent, such as bacterial or viral markers.

[00267] Because of the quantitative nature of detection reagents, the compositions and methods described herein can be used to quantitate target molecules whose abundance is indicative of a biological state or disease condition, for example, blood markers that are upregulated or downregulated as a result of a disease state.

[00268] In addition, the compositions and methods described herein can be used to provide prognostic information that assists in determining a course of treatment for a patient. For example, the amount of a particular marker for a tumor can be accurately quantified from even a small sample from a patient. For certain diseases like breast cancer, overexpression of certain genes, such as Her2-neu, indicate a more aggressive course of treatment will be needed.

[00269] In some embodiments, the compositions and methods described herein can be administered to a subject for in vivo diagnosis and/or monitoring of a disease or disorder. Specific devices or methods known in the art for the in vivo detection of fluorescence, e.g., from fluorophores or fluorescent proteins, include, but are not limited to, in vivo near-infrared fluorescence (see, e.g., Frangioni, *Curr. Opin. Chem. Biol,* 7:626-634 (2003)), the MaestroTM in vivo fluorescence imaging system (Cambridge Research & Instrumentation, Inc.; Woburn, Mass.), in vivo fluorescence imaging using a flying-spot scanner (see, e.g., Ramanujam et al, *IEEE Transactions on Biomedical Engineering,* 48:1034-1041 (2001), and the like. Other methods or devices for detecting an optical response include, without limitation, visual inspection, CCD cameras, video cameras, photographic film, laser-scanning devices,

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fluorometers, photodiodes, quantum counters, epifluorescence microscopes, scanning microscopes, flow cytometers, fluorescence microplate readers, or signal amplification using photomultiplier tubes.

[00270] Any device or method known in the art for detecting the radioactive emissions of radionuclides in a subject is suitable for use in various aspects described herein. For example, methods such as Single Photon Emission Computerized Tomography (SPECT), which detects the radiation from a single photon gamma-emitting radionuclide using a rotating gamma camera, and radionuclide scintigraphy, which obtains an image or series of sequential images of the distribution of a radionuclide in tissues, organs, or body systems using a scintillation gamma camera, may be used for detecting the radiation emitted from a radiolabeled aggregate. Positron emission tomography (PET) is another suitable technique for detecting radiation in a subject.

[00271] ***Analysis of Pathology Samples:*** RNA extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples is typically poor in quality (fragmented) and low in yield. This makes gene expression analysis of low-expressing genes in histology samples or archival pathology tissues extremely difficult and often completely infeasible. The detection reagents and methods described herein can fill this unmet need by allowing the analysis of very small quantities of low-quality total RNA.

[00272] To use detection reagents in such an application, total RNA can be extracted from formaldehyde- or paraformaldehyde-fixed paraffin-embedded tissue samples (or similar) using commercially available kits such as RecoverAll Total Nucleic Acid Isolation Kit (Ambion) following manufacturer's protocols. RNA in such samples is frequently degraded to small fragments (200 to 500 nucleotides in length), and many paraffin-embedded histology samples only yield tens of nanograms of total RNA. Small amounts (5 to 100 ng) of this fragmented total RNA can be used directly as target analyte upon contact with the detection reagents described herein, using the methods described herein described herein.

[00273] ***Screening Methods:*** The methods described herein can be used, among other things, for determining the effect of a perturbation, including chemical compounds, mutations, temperature changes, growth hormones, growth factors, disease, or a change in culture conditions, on various target molecules, thereby identifying target molecules whose presence, absence or levels are indicative of particular biological states. In one embodiment, some aspects described herein can be used to elucidate and discover components and pathways of disease

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states. For example, the comparison of quantities of target molecules present in a disease tissue with "normal" tissue allows the elucidation of important target molecules involved in the disease, thereby identifying targets for the discovery/screening of new drug candidates that can be used to treat disease.

[00274] ***Targeted Delivery Vehicles***: In some embodiments, the therapeutic and/or diagnostic agent can be encapsulated within the particles that provide conjugation sites for the probe reagents and nucleic acid labels. In such embodiments, the detection reagents and methods described herein can be used to deliver a therapeutic and/or diagnostic agent to cells where the probe reagents of the detection reagents bind. Nucleic acid labels of the detection reagents can be detected to monitor the location of drug administration.

[00275] ***Cell Lineage Tracking***: In some embodiments, the detection reagents described herein can be used to track cell lineage, e.g., in culture or in vivo. For example, the probe reagents of the detection reagents can bind to live cells of interest, allowing labeling and/or tracking of individual cell differentiation. Examples of such application include, but are not limited to, lineage tracking in stem cell differentiation, and lineage determination of dendritic cells and/or white blood cells. In some embodiments, these cells after lineage determination can be further used accordingly.

Kits comprising detection reagents

[00276] Kits for various assays are also provided herein. In some embodiments, a kit can comprise: (a) a plurality of nucleic acid labels of the detection reagents described herein; and (b) at least one coupling reagent required to conjugate the nucleic acid labels to probe reagents of interest. In such embodiments, users can attach the provided nucleic acid labels to their probe reagents of interest to form their own detection reagents described herein. Examples of the coupling reagent required for nucleic acid label-probe reagent conjugation can include any reagents that are generally used to perform any of the conjugation methods described herein, e.g., biotin and avidin-like molecules such as streptavidin or neutravidin. However, in alternative embodiments, the kit can comprise a plurality of the detection reagents described herein that are ready for use and thus no nucleic acid label-probe reagent conjugation steps are required to be performed by users prior to use.

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[00277] In some embodiments, the kit can further comprise at least one reagent, e.g., used in a readout method described herein. For example, if the readout method is sequencing-based, the kit can further comprise at least one agent used in sequencing-based readout. Alternatively, if the readout method is hybridization-based, the kit can further comprise at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

[00278] Accordingly, in some embodiments, a kit for hybridization-based readout can comprise: (a) a plurality of nucleic acid labels of the detection reagents described herein; (b) at least one reagent required to conjugate the nucleic acid labels to probe reagents of interest; and (c) at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature. In some embodiments, the kit can further comprise at least one reagent, e.g., used in the readout method.

[00279] In alternative embodiments, a kit for hybridization-based readout can comprise: (a) a plurality of the detection reagents described herein; (b) at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature; and (c) at least one reagent.

[00280] In any embodiments of the kit described herein, the plurality of the detection reagents can include one or more different kinds of the detection reagents. In some embodiments, it is desirable to provide different kinds of the detection reagents (e.g., different types of probe reagents, target binding domains, and/or target analytes) in separate containers. The number of different kinds of the detection reagents provided in the kit can be tailored for each application described herein. In some embodiments, the kit can comprise at least 2, at least 3, at least 4, at least 5, at least 6 at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100 or more different kinds of the detection reagents.

[00281] In any embodiments of the kit described herein , the kit can comprise a plurality of sets of decoder probes, e.g., e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10 or more sets of decoder probes, wherein each set of decoder probes can target a different pre-determined sequence of the nucleic acid

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label. In each set of the decoder probes, there can be about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more distinct populations of decoder probes. The decoder probes can be pre-labeled with at least one detectable label or unlabeled. In some embodiments where the decoder probes are not pre-labeled, the kit can further comprise one or more distinct detectable labels provided in separate containers for labeling the decoder probes. In some embodiments, the detectable label can be an optical label described herein.

[00282] Examples of a reagent, e.g., used in a readout method, can include, but are not limited to, a readout reagent, a wash buffer, a signal removal buffer, buffers for performing hybridization reactions, restriction endonucleases, nucleic acid ligases, and any combinations thereof.

[00283] In some embodiments, the detection reagents can be provided in a solution phase. In other embodiments, the detection reagents can be immobilized in a solid support or substrate.

[00284] By "substrate" or "solid support" or other grammatical equivalents herein is meant any material that can be modified to contain discrete individual sites appropriate for the attachment or association of beads and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, Teflon, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, optical fiber bundles, and a variety of other polymers. In general, the substrates allow optical detection and do not themselves appreciably fluoresce.

[00285] Generally the substrate is flat (planar), although as will be appreciated by those in the art, other configurations of substrates may be used as well; for example, three dimensional configurations can be used, for example by embedding the detection reagents in a porous block of plastic that allows sample access to the detection reagents and using a confocal microscope for detection. In some embodiments, the detection reagents can be placed on the inside surface of a tube, for flow-through sample analysis to minimize sample volume. Preferred substrates include optical fiber bundles, and flat planar substrates such as glass, polystyrene and other plastics and acrylics. In some embodiment, the solid support or substrate can be a multi-well plate.

[00286] In addition to the above mentioned components, the kit can include informational material. The informational material can be descriptive, instructional, marketing or other

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material that relates to the methods described herein and/or the use of the aggregates for the methods described herein. For example, the informational material describes methods for administering the detection reagents to a subject, and/or includes instructions to label decoder probes with detectable labels provided therein and/or instructions to conjugate at least one nucleic acid label to a probe reagent. The kit can also include a delivery device.

[00287] In some embodiments, the informational material can include instructions to administer the formulation in a suitable manner, e.g., in a suitable dose, dosage form, or mode of administration (e.g., a dose, dosage form, or mode of administration described herein). In another embodiment, the informational material can include instructions for identifying a suitable subject, e.g., a human, e.g., an adult human. The informational material of the kits is not limited in its form. In many cases, the informational material, e.g., instructions, is provided in printed matter, e.g., a printed text, drawing, and/or photograph, e.g., a label or printed sheet. However, the informational material can also be provided in other formats, such as Braille, computer readable material, video recording, or audio recording. In another embodiment, the informational material of the kit is a link or contact information, e.g., a physical address, email address, hyperlink, website, or telephone number, where a user of the kit can obtain substantive information about the formulation and/or its use in the methods described herein. Of course, the informational material can also be provided in any combination of formats.

[00288] In some embodiments, the kit contains separate containers, dividers or compartments for each component and informational material. For example, each different component can be contained in a bottle, vial, or syringe, and the informational material can be contained in a plastic sleeve or packet. In other embodiments, the separate elements of the kit are contained within a single, undivided container. For example, the formulation is contained in a bottle, vial or syringe that has attached thereto the informational material in the form of a label.

[00289] In some embodiments, the kit includes a plurality, e.g., a pack, of individual containers, each containing one or more unit dosage forms of the composition comprising the detection reagents. For example, the kit includes a plurality of syringes, ampules, foil packets, or blister packs, each containing a single unit dose of the formulation. The containers of the kits can be air tight and/or waterproof.

[00290] *Embodiments of the various aspects described herein can be illustrated by the following numbered paragraphs.*

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1. A method for detecting a plurality of analytes in a sample, comprising:

    a. contacting the sample with a composition comprising a plurality of detection reagents, wherein each subpopulation of the detection reagents targets at least one different analyte, and wherein each detection reagent comprises:

        at least one probe reagent targeting an analyte and at least one nucleic acid label comprising one or a plurality of pre-determined subsequences, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together; and wherein at least a portion of said one or the plurality of pre-determined subsequences form an identifier of said at least one probe reagent; and

    b. detecting in a temporally-sequential manner said one or the plurality of the pre-determined subsequences of said detection reagent, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents.

2. The method of paragraph 1, wherein said each subpopulation of the detection reagents targets a set of analytes (e.g., at least two analytes or more).

3. The method of paragraph 1 or 2, wherein the temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent is unique for each subpopulation of the detection reagents.

4. The method of any of paragraphs 1-3, wherein said detection reagents are present in a soluble phase.

5. The method of any of paragraphs 1-4, further comprising processing said sample before said contacting with said plurality of detection reagents.

6. The method of any of paragraphs 1-5, further comprising removing any unbound detection reagents before the detecting step (b).

7. The method of any of paragraphs 1-6, further comprising comparing said temporal order of the signal signatures with different identifiers of said at least one probe reagent,

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wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample.

8. The method of any of paragraphs 1-7, further comprising measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents.

9. The method of paragraph 8, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents indicates an amount of the analyte.

10. The method of paragraph 8 or 9, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents is used in identification of the subpopulation of the detection reagents.

11. The method of any of paragraphs 1-10, wherein said detecting of step (b) comprises sequencing.

12. The method of paragraph 11, wherein said sequencing is performed via ligation, hybridization, synthesis, amplification, or single-base extension.

13. The method of any of paragraphs 1-12 wherein said detecting of step (b) comprises hybridizing a decoder probe with said subsequence, wherein said decoder probe comprises a detectable label.

14. The method of any of paragraphs 1-13, wherein said detecting of step (b) comprises:

    a. hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes comprises a detectable label, each detectable label producing a signal signature;

    b. detecting said signal signature produced by the hybridization of said set of decoder probes;

    c. optionally removing said different signal signature produced by the hybridization of said set of decoder probes; and

    d. repeating steps (a) through (c) for other subsequences of said detection reagents, thereby producing a temporal order of the signal signatures corresponding to said each detection reagent.

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15. The method of paragraph 14, wherein said each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

16. The method of paragraph 14 or 15, wherein said each subpopulation of the decoder probes is at least partially or completely complementary to said subsequence of the detection reagents.

17. The method of any of paragraphs 14-16, wherein at least two or more subpopulations of the decoder probes are at least partially or completely complementary to the same subsequence of the detection reagents.

18. The method of any of paragraphs 14-17, wherein said removing step is performed by washing, heating, photobleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation or any combinations thereof.

19. The method of any of paragraphs 14-18, wherein said detectable label comprises or is an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule, a fluorescent protein, a quantum dot, Raman label, a chromophore, and any combinations thereof.

20. The method of any of paragraphs 14-19, wherein said detectable label comprises or is a colorimetric reagent.

21. The method of any of paragraphs 14-20, wherein said detectable label comprises or is a Raman label.

22. The method of any of paragraphs 1-21, wherein said signal signatures comprise or are optical signatures.

23. The method of paragraph 22, wherein said optical signatures comprise signatures of fluorescent color, visible light, no-color, Raman label, or any combinations thereof.

24. The method of paragraph 22, wherein said optical signatures comprise signatures of one or more fluorescent colors, one or more visible lights, one or more no-colors, one or more Raman labels, or any combinations thereof.

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25. The method of any of paragraphs 22-24, wherein said optical signatures are detected by optical imaging or spectroscopy.

26. The method of any of paragraphs 1-25, wherein said analytes are selected from the group consisting of antigens, receptors, proteins, peptides, sugars, glycoproteins, peptidoglycans, lipids, nucleic acids, oligonucleotides, cells, viruses, and any combinations thereof.

27. The method of any of paragraphs 1-26, wherein said nucleic acids are selected from the group consisting of cellular DNA or RNA, messenger RNA, microRNA, ribosomal RNA, and any combinations thereof.

28. The method of any of paragraphs 1-27, wherein said sample is a protein sample immobilized on a solid support.

29. The method of paragraph 28, wherein the solid support is a blotting membrane.

30. The method of any of paragraphs 1-27, wherein said sample is a biological sample.

31. The method of paragraph 30, wherein said biological sample comprises one or more cells, one or more tissues, one or more fluids or any combinations thereof.

32. The method of paragraph 30 or 31, wherein said biological sample comprises blood, sputum, cerebrospinal fluid, urine, saliva, sperm, sweat, mucus, nasal discharge, vaginal fluids or any combinations thereof.

33. The method of any of paragraphs 30-31, wherein the said biological sample comprises a biopsy, a surgically removed tissue, a swap or any combinations thereof.

34. The method of paragraphs 1-27, wherein said sample comprises an environmental sample, food, food byproduct, soil, an archaeological sample, an extraterrestrial sample, or any combinations thereof.

35. The method of any of paragraphs 1-34, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

36. The method of paragraph 35, wherein said linker is a bond.

37. The method of paragraph 35 or 36, wherein said linker is a linker molecule.

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38. The method of paragraph 37, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethylene glycol, crosslinker, or any combinations thereof.

39. The method of any of paragraphs 35-38, wherein said linker is a particle.

40. The method of paragraph 39, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.

41. The method of paragraph 40, wherein said particle is a nanoparticle.

42. The method of any of paragraphs 39-41, wherein said particle is modified.

43. The method of any of paragraphs 39-42, wherein said particle is coated with streptavidin or a derivative thereof.

44. The method of any of paragraphs 39-43, wherein said particle is modified with at least one functional group.

45. The method of paragraph 44, wherein said at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.

46. The method of any of paragraphs 35-45, wherein said linker is multivalent.

47. The method of paragraph 46, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.

48. The method of any of paragraphs 1-47, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.

49. The method of any of paragraphs 1-48, wherein said at least one probe reagent is modified.

50. The method of any of paragraphs 1-49, wherein said at least one probe reagent is biotinylated.

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51. The method of any of paragraphs 1-50, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.

52. The method of any of paragraphs 1-51, wherein said at least one nucleic acid label is modified.

53. The method of any of paragraphs 1-52, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.

54. The method of any of paragraphs 1-53, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.

55. The method of paragraph 54, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of a small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.

56. The method of any of paragraphs 1-55, wherein each of said plurality of the pre-determined subsequences comprises at least one base.

57. The method of any of paragraphs 1-56, wherein each of said plurality of the pre-determined subsequences comprises from 1 to 100 nucleobases.

58. The method of any of paragraphs 1-57, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.

59. The method of paragraph 58, wherein said sequence linker is a bond.

60. The method of any of paragraphs 58-59, wherein said sequence linker is a nucleotidic linker.

61. The method of paragraph 60, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin or any combinations thereof.

62. The method of paragraph 60 or 61, wherein said nucleotidic linker is at least one nucleotide long.

63. The method of any of paragraphs 1-62, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

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64. The method of any of paragraphs 1-62, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.

65. The method of any of paragraphs 1-62, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.

66. The method of any of paragraphs 1-65, wherein the method is adapted for use in immunofluorescence.

67. The method of any of paragraphs 1-66, wherein the method is adapted for use in immunohistochemistry.

68. The method of any of paragraphs 1-67, wherein the method is adapted for use in fluorescence in situ hybridization.

69. The method of any of paragraphs 1-68, wherein the method is adapted for use in western blot.

70. A detection reagent comprising at least one probe reagent and at least one nucleic acid label,

wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner;

wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and

wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

71. The detection reagent of paragraph 70, wherein the detection reagent is present in a soluble phase.

72. The detection reagent of paragraph 70 or 71, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

73. The detection reagent of paragraph 72, wherein said linker is a bond.

74. The detection reagent of paragraph 72 or 73, wherein said linker is a linker molecule.

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75. The detection reagent of paragraph 74, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne glycol, crosslinker or combination thereof.

76. The detection reagent of any of paragraphs 72-75, wherein said linker is a particle.

77. The detection reagent of paragraph 76, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.

78. The detection reagent of paragraph 76 or 77, wherein said particle is a nanoparticle.

79. The detection reagent of any of paragraphs 76-78, wherein said particle is modified.

80. The detection reagent of any of paragraphs 76-79, wherein said particle is coated with streptavidin or a derivative thereof.

81. The detection reagent of any of paragraphs 76-80, wherein said particle is modified with at least one functional group.

82. The detection reagent of paragraph 81, wherein the at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.

83. The detection reagent of any of paragraphs 72-82, wherein said linker is multivalent.

84. The detection reagent of paragraph 83, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.

85. The detection reagent of any of paragraphs 70-84, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.

86. The detection reagent of any of paragraphs 70-85, wherein said at least one probe reagent is modified.

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87. The detection reagent of any of paragraphs 70-86, wherein said at least one probe reagent is biotinylated.

88. The detection reagent of any of paragraphs 70-87, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.

89. The detection reagent of any of paragraphs 70-88, wherein said at least one nucleic acid label is modified.

90. The detection reagent of any of paragraphs 70-89, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.

91. The detection reagent of any of paragraphs 70-90, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.

92. The detection reagent of paragraph 91, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.

93. The detection reagent of any of paragraphs 70-92, wherein said at least one nucleic acid label comprises a plurality of pre-determined subsequences.

94. The detection reagent of any of paragraphs 70-93, wherein each of said plurality of predetermined subsequences comprises at least one base.

95. The detection reagent of any of paragraphs 70-94, wherein each of said plurality of predetermined subsequences comprises from 1 to 100 nucleobases.

96. The detection reagent of any of paragraphs 70-95, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.

97. The detection reagent of paragraph 96, wherein said sequence linker is a bond.

98. The detection reagent of any of paragraphs 96-97, wherein said sequence linker is a nucleotidic linker.

99. The detection reagent of paragraph 98, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

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100. The detection reagent of paragraph 98 or 99, wherein said nucleotidic linker is at least one nucleotide long.

101. The detection reagent of any of paragraphs 70-100, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

102. The detecton reagent of any of paragraphs 70-101, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.

103. The detection reagent of any of paragraphs 70-102, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.

104. The detection reagent of any of paragraphs 70-103, wherein the detection reagent is adapted for use in immunofluorescence.

105. The detection reagent of any of paragraphs 70-104, wherein the detection reagent is adapted for use in immunohistochemistry.

106. The detection reagent of any of paragraphs 70-105, wherein the detection reagent is adapted for use in fluorescence in situ hybridization.

107. The detection reagent of any of paragraphs 70-106, wherein the detection reagent is adapted for use in western blot.

108. A kit comprising:

a. a plurality of the detection reagents of any of paragraphs 70-107; and

b. at least one reagent.

109. The kit of paragraph 108, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

110. The kit of paragraph 108 or 109, wherein said detection reagents are present in a soluble phase.

111. The kit of any of paragraphs 108-110, wherein said detection reagents are immobilized in a multi-well plate.

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112. The kit of any of paragraphs 108-111, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

113. A kit comprising:

a. a plurality of the nucleic acid labels of the detection reagents of any of paragraphs 70-107;

b. at least one coupling agent that allows a user to conjugate the nucleic acid labels to the user's probe reagents of interest, thereby forming the detection reagents comprising the user's probe reagents of interest; and

c. at least one reagent.

114. The kit of paragraph 113, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

115. The kit of paragraph 113 or 114, wherein the detection reagents comprising the user's probe reagents of interest are present in a soluble phase.

116. The kit of any of paragraphs 113-115, wherein the detection reagents comprising the user's probe reagents of interest are immobilized in a multi-well plate.

117. The kit of any of paragraphs 113-116, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

Some selected definitions

[00291] Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments of the aspects described herein, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Further, unless

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otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

[00292] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not. Additionally, the term "comprising" or "comprises" includes "consisting essentially of" and "consisting of."

[00293] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00294] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00295] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean ±1%.

[00296] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise.

[00297] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term "comprises" means "includes." The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[00298] As used herein, the term "identifier" generally refers to a unique expression to distinguish variations from one to another among a class of substances, items, or objects. In particular embodiments, the term "identifier" as used herein refers to association of a unique pre-determined subsequence to a specific probe reagent, thus conferring the presence and identity of the probe reagent when the pre-determined subsequence is detected.

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[00299] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) above or below a reference level. The term refers to statistical evidence that there is a difference. It is defined as the probability of making a decision to reject the null hypothesis when the null hypothesis is actually true. The decision is often made using the p-value.

[00300] As used herein, the term "substantially" means a proportion of at least about 60%, or preferably at least about 70% or at least about 80%, or at least about 90%, at least about 95%, at least about 97% or at least about 99% or more, or any integer between 70% and 100%. In some embodiments, the term "substantially" means a proportion of at least about 90%, at least about 95%, at least about 98%, at least about 99% or more, or any integer between 90% and 100%. In some embodiments, the term "substantially" can include 100%.

[00301] The term "sphere" means a particle having an aspect ratio of at most 3:1. The term "aspect ratio" means the ratio of the longest axis of an object to the shortest axis of the object, where the axes are not necessarily perpendicular.

[00302] The term "rod" means a particle having a longest dimension of at most 5000 nm, and having an aspect ratio of from 3:1 to 20:1.

[00303] The term "prism" means a particle having at least two non-parallel faces connected by a common edge.

[00304] As used herein, the term "pharmaceutically acceptable carrier" refers to a pharmaceutically-acceptable material, composition or vehicle for administration of the detection reagents. Pharmaceutically acceptable carriers include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like which are compatible with the activity of the detection reagents and are physiologically acceptable to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and

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polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (22) C2-C12 alchols, such as ethanol; and (23) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservative and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" are used interchangeably herein.

[00305] As used here, the term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00306] As used herein, the term "therapeutic agent" refers to a biological or chemical agent used for treatment, curing, mitigating, or preventing deleterious conditions in a subject. The term "therapeutic agent" also includes substances and agents for combating a disease, condition, or disorder of a subject, and includes drugs, diagnostics, and instrumentation. "Therapeutic agent" also includes anything used in medical diagnosis, or in restoring, correcting, or modifying physiological functions. The terms "therapeutic agent" and "pharmaceutically active agent" are used interchangeably herein.

[00307] A therapeutic agent can be selected according to the treatment objective and biological action desired. Thus, a therapeutic agent can be selected from any class suitable for the therapeutic objective. Further, the therapeutic agent may be selected or arranged to provide therapeutic activity over a period of time.

[00308] Exemplary pharmaceutically active compound include, but are not limited to, those found in Harrison's Principles of Internal Medicine, 13th Edition, Eds. T.R. Harrison McGraw-Hill N.Y., NY; Physicians Desk Reference, 50th Edition, 1997, Oradell New Jersey, Medical Economics Co.; Pharmacological Basis of Therapeutics, 8th Edition, Goodman and Gilman, 1990; United States Pharmacopeia, The National Formulary, USP XII NF XVII, 1990; current

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edition of Goodman and Oilman's The Pharmacological Basis of Therapeutics; and current edition of The Merck Index, the complete content of all of which are herein incorporated in its entirety.

[00309] Exemplary pharmaceutically active agents include, but are not limited to, steroids and nonsteroidal anti-inflammatory agents, antirestenotic drugs, antimicrobial agents, angiogenic factors, calcium channel blockers, thrombolytic agents, antihypertensive agents, anti-coagulants, antiarrhythmic agents, cardiac glycosides, and the like.

[00310] In some embodiments, the therapeutic agent is selected from the group consisting of salicylic acid and derivatives (aspirin), para-aminophenol and derivatives (acetaminophen), arylpropionic acids (ibuprofen), corticosteroids, histamine receptor antagonists and bradykinin receptor antagonists, leukotriene receptor antagonists, prostaglandin receptor antagonists, platelet activating factor receptor antagonists, sulfonamides, trimethoprim-sulfamethoxazole, quinolones, penicillins, cephalosporin, basic fibroblast growth factor (FGF), acidic fibroblast growth factor, vascular endothelial growth factor, angiogenic transforming growth factor alpha and beta, tumor necrosis factor, angiopoietin, platelet-derived growth factor, dihydropyridines (e.g., nifedipine, benzothiazepines such as dilitazem, and phenylalkylamines such as verapamil), urokinase plasminogen activator, urokinase, streptokinase, angiotensin converting enzyme (ACE) inhibitors, spironolactone, tissue plasminogen activator (tPA), diuretics, thiazides, antiadrenergic agents, clonidine, propanolol, angiotensin-converting enzyme inhibitors, captopril, angiotensin receptor antagonists, losartan, calcium channel antagonists, nifedine, heparin, warfarin, hirudin, tick anti-coagulant peptide, and low molecular weight heparins such as enoxaparin, lidocaine, procainamide, encainide, flecanide, beta adrenergic blockers, propranolol, amiodarone, verpamil, diltiazem, nickel chloride, cardiac glycosides, angiotensin converting enzyme inhibitors, angiotensin receptor antagonists, nitrovasodilators, hypolipidemic agents (e.g., nicotinic acid, probucol, etc.), bile acid-binding resins (e.g., cholestyramine, and fibric acid derivatives e.g., clofibrate), HMG CoA reductase inhibitors, HMG CoA synthase inhibitors, squalene synthase inhibitors, squalene epoxidase inhibitors, statins (e.g., lovastatin, cerivastatin, fluvastatin, pravastatin, simvaststin, etc.), anti-psychotics, SSRIs, antiseizure medication, contraceptives, systemic and local analgesics (chronic pain, bone growth/remodeling factors (osteoblast/osteoclast recruiting and stimulating factors), neurotransmitters (L-DOPA, Dopamine, neuropeptides), emphysema drugs, TGF-beta), rapamycin, naloxone, paclitaxel,

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amphotericin, Dexamethasone, flutamide, vancomycin, phenobarbital, cimetidine, atenolol, aminoglycosides, hormones (e.g., thyrotropin-releasing hormone, p-nitrophenyl beta-cellopentaosideand luteinizing hormone-releasing hormone), vincristine, amiloride, digoxin, morphine, procainamide, quinidine, quinine, ranitidine, triamterene, trimethoprim, vancomycin, aminoglycosides, and penicillin, and pharmaceutically acceptable salts thereof.

[00311] As used herein, the term "fluorescent color" refers to a color emitted by any fluorophore (e.g., fluorescent dye, fluorescent particles such as quantum dots, and/or fluorescent proteins) upon light excitation and/or electromagnetic radiation. Fluorescent dye as disclosed herein refers to a dye which exhibits energy and is visible under illumination at a predefined wavelength. Numerous fluorescent molecules are commercially available and can be adapted for use in the methods, detection reagents and kits as disclosed herein, and include those from Molecular Probes, Sigma and similar other commercial sources. In some embodiments, a "fluorescent color" can be produced by phosphorescence. In some embodiments, a "fluorescent color" can be produced by luminescence (including bioluminescence).

[00312] To the extent not already indicated, it will be understood by those of ordinary skill in the art that any one of the various embodiments herein described and illustrated may be further modified to incorporate features shown in any of the other embodiments disclosed herein.

[00313] The following examples illustrate some embodiments and aspects of the invention. It will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be performed without altering the spirit or scope of the invention, and such modifications and variations are encompassed within the scope of the invention as defined in the claims which follow. The following examples do not in any way limit the invention.

EXAMPLES

Example 1: Exemplary hybridization-based detection methods of detection reagents

[00314] A solution suspension of streptavidin-coated Dynabeads (of about 1 μm), each conjugated to one of six nucleic acid labels and a corresponding probe reagent specific for a target analyte, wherein each of the nucleic acid labels comprised at least one pre-determined subsequence, e.g., a first (e.g., A1, A2, and A3) and a second (B1, B2, and B3) pre-determined subsequence, was prepared. The nucleic acid labels can comprise a nucleic acid sequence of any

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length. In some embodiments, the nucleic acid labels can comprise at least 15 nucleotides, at least 20 nucleotides, at least 22 nucleotides, or at least 24 nucleotides. A sample containing multiple target analytes was contacted with the target analyte-specific Dynabeads, so that the Dynabeads would bind to the respective target analyte on the sample. Any unbound Dynabeads was then removed, e.g., by washing. In the first readout, a set of three decoder probes, each with a distinct sequence complementary to the first pre-determined subsequences (e.g., A1*, A2*, and A3*) and a corresponding optical label (e.g., selected from red, green or blank), was added to the sample with bound Dynabeads, and then imaged with fluorescent microscopy (**Fig. 6A**). The fluorescent signal was then removed, e.g., by photobleaching, from the previous stage before the next readout. In the second readout, a different set of three decoder probes, each with a distinct complementary sequence to the second pre-determined subsequences (e.g., B1*, B2*, B3*) and a corresponding optical label (e.g., selected from red, green blank), was added to the photobleached sample, and then imaged with fluorescent microscopy (**Fig. 6B**). The temporal sequence of the optical signatures obtained from each bead can then be analyzed to determine the identity of the probe and thus the presence of the corresponding target analyte on the sample. Based on the intensity and/or coverage of the optical signals, the amount of the target analyte can also be determined.

[00315] By way of example only, a biotinylated anti-*C. albicans* antibody (e.g., an commercially-available antibody from Pierce Antibodies PA1-27145) was conjugated with a plurality of different biotinylated nucleic acid labels (e.g., 8 different biotinylated SeqTag label sequences) using any conjugation methods known in the art. In one embodiment, the biotinylated anti-*C. albicans* antibody was conjugated with a plurality of different biotinylated nucleic acid labels (e.g., biotinylated SeqTag label sequences) using a streptavidin-like protein (e.g., Neutravidin) as a bridge. The SeqTag label sequences are shown in Table 5. The SeqTag label sequences can comprise at least about 24 nucleotides. In some embodiments, the SeqTag label sequences can comprise DNA, RNA or a combination thereof. Each conjugate was incubated with a sample comprising *C. albicans*, washed, and readout using any readout method as described herein, e.g., the displacement-hybridization method.

Table 5. DNA sequences of exemplary SeqTag labels

SEQ ID NO:	SeqTag label	DNA Sequence

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59	SeqTag 1	5'-Biotin – TTTCGCTTTCTGTAATGGAGTGGA – 3'
60	SeqTag 2	5'-Biotin – TTTCGCTTTAGCCTAAGTGAAATC – 3'
61	SeqTag 3	5'-Biotin – TTTCGCTTTTTTGGGGAAAAGACA – 3'
62	SeqTag 4	5'-Biotin – TTTCGCTTTTAGGCATTAGCATTG – 3'
63	SeqTag 5	5'-Biotin – TTTCGCTTTGGAAGCACCTATTCC – 3'
64	SeqTag 6	5'-Biotin – TTTCGCTTTCTGGAGAAAGGGCCA – 3'
65	SeqTag 7	5'-Biotin – TTTCGCTTTCGGTTCCAAAGACAC – 3'
66	SeqTag 8	5'-Biotin – TTTCGCTTTGAAGCCGGTTATAGC – 3'

[00316] During the displacement hybridization method, by way of example only, as shown in **Fig. 7**, the yeast was incubated in readout step 1 with a mixture of all three "set 1" decoder probes, followed by an incubation with a mixture of the "set 2" decoder probes and the "set 1 displacers" (which are nucleic acid sequences to remove "set 1" fluorescence), and similarly with appropriate sets of decoder probes and displacers for steps 3 and 4. The decoder probes can comprise a nucleic acid sequence (e.g., DNA, RNA or a combination thereof) and a detection label, e.g., at its 5' end. The nucleic acid sequence of the decoder probes can be of any length. In some embodiments, the decoder probes can comprise at least about 10 nucleotides, at least about 12 nucleotides, at least about 14 nucleotides, at least about 16 nucleotides, at least about 18 nucleotides, at least about 20 nucleotides or longer. Detection labels can be any art-recognized label described herein, e.g., fluorescent detection labels such as FAM, Cy3, and C5, or any labels described herein. Probe displacers, as described earlier, are nucleic acid sequences that are used to remove or displace the previous decoder probes hybridized to the nucleic acid labels (e.g., SeqTag labels) such that a different set of decoder probes can be added and hybridized to the nucleic acid labels. The probe displacers can have a nucleic acid sequence of any length. In some embodiments, the nucleic acid sequence of the probe displacers can be longer than that of the decoder probes. In some embodiments, the probe displacers can comprise at least about 12 nucleotides, at least about 14 nucleotides, at least about 16 nucleotides, at least about 18 nucleotides, at least about 20 nucleotides, at least about 21 nucleotides or longer. Tables 2 and 3 show DNA sequences of exemplary decoder probes (or readout probes) and probe displacers, respectively. As seen in **Fig. 7**, each biotinylated anti-*C albicans* antibody conjugated with a different SeqTag label sequence properly stained the yeast and fluorescenced only as designated by its SeqTag label with appropriate sets of decoder probes and probe displacer in each readout step.

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Table 6. DNA sequences of exemplary decoder probes

SEQ ID NO:	Readout probe	DNA Sequence
67	Set 1 FAM	5'-FAM – TTTTCCACTCCATTACAG – 3'
68	Set 1 Cy3	5'-Cy3 – TTTGATTTCACTTAGGCT – 3'
69	Set 1 Cy5	5'-Cy5 – TTTTGTCTTTTCCCCAAA – 3'
70	Set 2 FAM	5'-FAM – TTTCAATGCTAATGCCTA – 3'
71	Set 2 Cy3	5'-Cy3 – TTTGGAATAGGTGCTTCC – 3'
72	Set 2 Cy5	5'-Cy5 – TTTTGGCCCTTTCTCCAG – 3'
73	Set 3 FAM	5'-FAM – TTTGTGTCTTTGGAACCG – 3'
74	Set 3 Cy3	5'-Cy3 – TTTGCTATAACCGGCTTC – 3'

Table 7. DNA sequences of exemplary probe displacers

SEQ ID NO:	Probe Displacer	DNA Sequence
75	Set 1 FAM-Displacer	5' – TCCACTCCATTACAGAAAGCG – 3'
76	Set 1 Cy3-Displacer	5' – GATTTCACTTAGGCTAAAGCG – 3'
77	Set 1 Cy5-Displacer	5' – TGTCTTTTCCCCAAAAAAGCG – 3'
78	Set 2 FAM-Displacer	5' – CAATGCTAATGCCTAAAAGCG – 3'
79	Set 2 Cy3-Displacer	5' – GGAATAGGTGCTTCCAAAGCG – 3'
80	Set 2Cy5-Displacer	5' – TGGCCCTTTCTCCAGAAAGCG – 3'
81	Set 3 FAM-Displacer	5' –TGTGTCTTTGGAACCGAAAGCG – 3'
82	Set 3 Cy3-Displacer	5' – GCTATAACCGGCTTCAAAGCG – 3'

[00317] Content of all patents and other publications identified herein is expressly incorporated herein by reference for all purposes. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

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CLAIMS

What is claimed is:

1. A method for detecting a plurality of analytes in a sample, comprising:

    a. contacting the sample with a composition comprising a plurality of detection reagents, wherein each subpopulation of the detection reagents targets at least one different analyte, and wherein each detection reagent comprises:

        at least one probe reagent targeting an analyte and at least one nucleic acid label comprising one or a plurality of pre-determined subsequences, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together; and wherein at least a portion of said one or the plurality of pre-determined subsequences form an identifier of said at least one probe reagent; and

    b. detecting in a temporally-sequential manner said one or the plurality of the pre-determined subsequences of said detection reagent, wherein said detection of the subsequences each generates a signal signature corresponding to said subsequence, and wherein a temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent identifies a subpopulation of the detection reagents.

2. The method of claim 1, wherein said each subpopulation of the detection reagents targets a set of analytes.

3. The method of claim 1 or 2, wherein the temporal order of the signal signatures corresponding to said one or the plurality of the subsequences of said detection reagent is unique for each subpopulation of the detection reagents.

4. The method of any of claims 1-3, wherein said detection reagents are present in a soluble phase.

5. The method of any of claims 1-4, further comprising processing said sample before said contacting with said plurality of detection reagents.

6. The method of any of claims 1-5, further comprising removing any unbound detection reagents before the detecting step (b).

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7. The method of any of claims 1-6, further comprising comparing said temporal order of the signal signatures with different identifiers of said at least one probe reagent, wherein an agreement between the temporal order of the signal signatures and a particular identifier of said at least one probe reagent identifies the analyte in the sample.

8. The method of any of claims 1-7, further comprising measuring the intensity of the signal signatures generated from each subpopulation of the detection reagents.

9. The method of claim 8, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents indicates an amount of the analyte.

10. The method of claim 8 or 9, wherein the intensity of the signal signatures generated from each subpopulation of the detection reagents is used in identification of the subpopulation of the detection reagents.

11. The method of any of claims 1-10, wherein said detecting of step (b) comprises sequencing.

12. The method of claim 11, wherein said sequencing is performed via ligation, hybridization, synthesis, amplification, or single-base extension.

13. The method of any of claims 1-12 wherein said detecting of step (b) comprises hybridizing a decoder probe with said subsequence, wherein said decoder probe comprises a detectable label.

14. The method of any of claims 1-13, wherein said detecting of step (b) comprises:

    a. hybridizing a set of decoder probes with a subsequence of the detection reagents, wherein each subpopulation of the decoder probes comprises a detectable label, each detectable label producing a signal signature;

    b. detecting said signal signature produced by the hybridization of said set of decoder probes;

    c. optionally removing said different signal signature produced by the hybridization of said set of decoder probes; and

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d. repeating steps (a) through (c) for other subsequences of said detection reagents, thereby producing a temporal order of the signal signatures corresponding to said each detection reagent.

15. The method of claim 14, wherein said each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

16. The method of claim 14 or 15, wherein said each subpopulation of the decoder probes is at least partially or completely complementary to said subsequence of the detection reagents.

17. The method of any of claims 14-16, wherein at least two or more subpopulations of the decoder probes are at least partially or completely complementary to the same subsequence of the detection reagents.

18. The method of any of claims 14-17, wherein said removing step is performed by washing, heating, photobleaching, displacement, cleavage, enzymatic digestion, quenching, chemical degradation, bleaching, oxidation or any combinations thereof.

19. The method of any of claims 14-18, wherein said detectable label comprises or is an optical label selected from the group consisting of a small-molecule dye, a fluorescent molecule, a fluorescent protein, a quantum dot, Raman label, a chromophore, and any combinations thereof.

20. The method of any of claims 14-19, wherein said detectable label comprises or is a colorimetric reagent.

21. The method of any of claims 14-20, wherein said detectable label comprises or is a Raman label.

22. The method of any of claims 1-21, wherein said signal signatures comprise or are optical signatures.

23. The method of claim 22, wherein said optical signatures comprise signatures of fluorescent color, visible light, no-color, Raman label, or any combinations thereof.

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24. The method of claim 22, wherein said optical signatures comprise signatures of one or more fluorescent colors, one or more visible lights, one or more no-colors, one or more Raman labels, or any combinations thereof.

25. The method of any of claims 22-24, wherein said optical signatures are detected by optical imaging or spectroscopy.

26. The method of any of claims 1-25, wherein said analytes are selected from the group consisting of antigens, receptors, proteins, peptides, sugars, glycoproteins, peptidoglycans, lipids, nucleic acids, oligonucleotides, cells, viruses, and any combinations thereof.

27. The method of any of claims 1-26, wherein said nucleic acids are selected from the group consisting of cellular DNA or RNA, messenger RNA, microRNA, ribosomal RNA, and any combinations thereof.

28. The method of any of claims 1-27, wherein said sample is a protein sample immobilized on a solid support.

29. The method of claim 28, wherein the solid support is a blotting membrane.

30. The method of any of claims 1-27, wherein said sample is a biological sample.

31. The method of claim 30, wherein said biological sample comprises one or more cells, one or more tissues, one or more fluids or any combinations thereof.

32. The method of claim 30 or 31, wherein said biological sample comprises blood, sputum, cerebrospinal fluid, urine, saliva, sperm, sweat, mucus, nasal discharge, vaginal fluids or any combinations thereof.

33. The method of any of claims 30-31, wherein the said biological sample comprises a biopsy, a surgically removed tissue, a swap or any combinations thereof.

34. The method of claims 1-27, wherein said sample comprises an environmental sample, food, food byproduct, soil, an archaeological sample, an extraterrestrial sample, or any combinations thereof.

35. The method of any of claims 1-34, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

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36. The method of claim 35, wherein said linker is a bond.

37. The method of claim 35 or 36, wherein said linker is a linker molecule.

38. The method of claim 37, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethylene glycol, crosslinker, or any combinations thereof.

39. The method of any of claims 35-38, wherein said linker is a particle.

40. The method of claim 39, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.

41. The method of claim 40, wherein said particle is a nanoparticle.

42. The method of any of claims 39-41, wherein said particle is modified.

43. The method of any of claims 39-42, wherein said particle is coated with streptavidin or a derivative thereof.

44. The method of any of claims 39-43, wherein said particle is modified with at least one functional group.

45. The method of claim 44, wherein said at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.

46. The method of any of claims 35-45, wherein said linker is multivalent.

47. The method of claim 46, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.

48. The method of any of claims 1-47, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.

49. The method of any of claims 1-48, wherein said at least one probe reagent is modified.

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50. The method of any of claims 1-49, wherein said at least one probe reagent is biotinylated.

51. The method of any of claims 1-50, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.

52. The method of any of claims 1-51, wherein said at least one nucleic acid label is modified.

53. The method of any of claims 1-52, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.

54. The method of any of claims 1-53, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.

55. The method of claim 54, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of a small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.

56. The method of any of claims 1-55, wherein each of said plurality of the pre-determined subsequences comprises at least one base.

57. The method of any of claims 1-56, wherein each of said plurality of the pre-determined subsequences comprises from 1 to 100 nucleobases.

58. The method of any of claims 1-57, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.

59. The method of claim 58, wherein said sequence linker is a bond.

60. The method of any of claims 58-59, wherein said sequence linker is a nucleotidic linker.

61. The method of claim 60, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin or any combinations thereof.

62. The method of claim 60 or 61, wherein said nucleotidic linker is at least one nucleotide long.

63. The method of any of claims 1-62, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

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64. The method of any of claims 1-62, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.

65. The method of any of claims 1-62, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.

66. The method of any of claims 1-65, wherein the method is adapted for use in immunofluorescence.

67. The method of any of claims 1-66, wherein the method is adapted for use in immunohistochemistry.

68. The method of any of claims 1-67, wherein the method is adapted for use in fluorescence in situ hybridization.

69. The method of any of claims 1-68, wherein the method is adapted for use in western blot.

70. A detection reagent comprising at least one probe reagent and at least one nucleic acid label,

wherein said at least one nucleic acid label comprises at least one pre-determined subsequence to be detected in a temporally-sequential manner;

wherein said at least one pre-determined subsequence forms an identifier of said at least one probe reagent; and

wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together.

71. The detection reagent of claim 70, wherein the detection reagent is present in a soluble phase.

72. The detection reagent of claim 70 or 71, wherein said at least one probe reagent and said at least one nucleic acid label are conjugated together by at least one linker.

73. The detection reagent of claim 72, wherein said linker is a bond.

74. The detection reagent of claim 72 or 73, wherein said linker is a linker molecule.

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75. The detection reagent of claim 74, wherein said linker molecule is a polymer, sugar, nucleic acid, peptide, protein, hydrocarbon, lipid, polyethelyne glycol, crosslinker or combination thereof.

76. The detection reagent of any of claims 72-75, wherein said linker is a particle.

77. The detection reagent of claim 76, wherein said particle is selected from a group consisting of a gold nanoparticle, a magnetic bead or nanoparticle, a polystyrene bead, a nanotube, a nanowire, a microparticle, and any combinations thereof.

78. The detection reagent of claim 76 or 77, wherein said particle is a nanoparticle.

79. The detection reagent of any of claims 76-78, wherein said particle is modified.

80. The detection reagent of any of claims 76-79, wherein said particle is coated with streptavidin or a derivative thereof.

81. The detection reagent of any of claims 76-80, wherein said particle is modified with at least one functional group.

82. The detection reagent of claim 81, wherein the at least one functional group is selected from the group consisting of amine, carboxyl, hydroxyl, aldehyde, ketone, tosyl, silanol, chlorine, hydrazine, hydrazide, photoreactive groups, and any combinations thereof.

83. The detection reagent of any of claims 72-82, wherein said linker is multivalent.

84. The detection reagent of claim 83, wherein when the multivalent linker is an avidin-like molecule, both the probe reagent and the nucleic acid label are biotinylated.

85. The detection reagent of any of claims 70-84, wherein said at least one probe reagent is selected from the group consisting of a nucleic acid, an antibody or a portion thereof, an antibody-like molecule, an enzyme, a cell, an antigen, a small molecule, a protein, a peptide, a peptidomimetic, a sugar, a carbohydrate, a lipid, a glycan, a glycoprotein, an aptamer, and any combinations thereof.

86. The detection reagent of any of claims 70-85, wherein said at least one probe reagent is modified.

87. The detection reagent of any of claims 70-86, wherein said at least one probe reagent is biotinylated.

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88. The detection reagent of any of claims 70-87, wherein said at least one nucleic acid label is single-stranded, double-stranded, partially double-stranded, a hairpin, linear, circular, branched, a concatemer, or any combinations thereof.

89. The detection reagent of any of claims 70-88, wherein said at least one nucleic acid label is modified.

90. The detection reagent of any of claims 70-89, wherein said at least one nucleic acid label is designed for minimal cross-hybridization of bases with each other.

91. The detection reagent of any of claims 70-90, wherein said at least one nucleic acid label is conjugated to at least one detectable molecule.

92. The detection reagent of claim 91, wherein said at least one detectable molecule is an optical molecule selected from the group consisting of small-molecule dye, a fluorescent protein, a quantum dot, a Raman label, a chromophore, and any combinations thereof.

93. The detection reagent of any of claims 70-92, wherein said at least one nucleic acid label comprises a plurality of pre-determined subsequences.

94. The detection reagent of any of claims 70-93, wherein each of said plurality of predetermined subsequences comprises at least one base.

95. The detection reagent of any of claims 70-94, wherein each of said plurality of predetermined subsequences comprises from 1 to 100 nucleobases.

96. The detection reagent of any of claims 70-95, wherein said plurality of the pre-determined subsequences are conjugated together by at least one sequence linker.

97. The detection reagent of claim 96, wherein said sequence linker is a bond.

98. The detection reagent of any of claims 96-97, wherein said sequence linker is a nucleotidic linker.

99. The detection reagent of claim 98, wherein said nucleotidic linker is single-stranded, double-stranded, partially double-stranded, a hairpin, or any combinations thereof.

100. The detection reagent of claim 98 or 99, wherein said nucleotidic linker is at least one nucleotide long.

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101. The detection reagent of any of claims 70-100, wherein said detection reagent comprises one probe reagent and a plurality of nucleic acid labels.

102. The detecton reagent of any of claims 70-101, wherein said detection reagent comprises a plurality of probe reagents and a nucleic acid label.

103. The detection reagent of any of claims 70-102, wherein said detection reagent comprises a plurality of probe reagents and a plurality of nucleic acid labels.

104. The detection reagent of any of claims 70-103, wherein the detection reagent is adapted for use in immunofluorescence.

105. The detection reagent of any of claims 70-104, wherein the detection reagent is adapted for use in immunohistochemistry.

106. The detection reagent of any of claims 70-105, wherein the detection reagent is adapted for use in fluorescence in situ hybridization.

107. The detection reagent of any of claims 70-106, wherein the detection reagent is adapted for use in western blot.

108. A kit comprising:

a. a plurality of the detection reagents of any of claims 70-107; and

b. at least one reagent.

109. The kit of claim 108, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

110. The kit of claim 108 or 109, wherein said detection reagents are present in a soluble phase.

111. The kit of any of claims 108-110, wherein said detection reagents are immobilized in a multi-well plate.

112. The kit of any of claims 108-111, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

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113. A kit comprising:

a. a plurality of the nucleic acid labels of the detection reagents of any of claims 70-107;

b. at least one coupling agent that allows a user to conjugate the nucleic acid labels to the user's probe reagents of interest, thereby forming the detection reagents comprising the user's probe reagents of interest; and

c. at least one reagent.

114. The kit of claim 113, wherein the kit further comprises at least one set of decoder probes complementary to at least a portion of subsequences of the detection reagents, wherein each subpopulation of the decoder probes comprises a different detectable label, each different detectable label producing a different signal signature.

115. The kit of claim 113 or 114, wherein the detection reagents comprising the user's probe reagents of interest are present in a soluble phase.

116. The kit of any of claims 113-115, wherein the detection reagents comprising the user's probe reagents of interest are immobilized in a multi-well plate.

117. The kit of any of claims 113-116, wherein said at least one reagent is selected from the group consisting of a readout reagent, a wash buffer, a signal removal buffer, and any combinations thereof.

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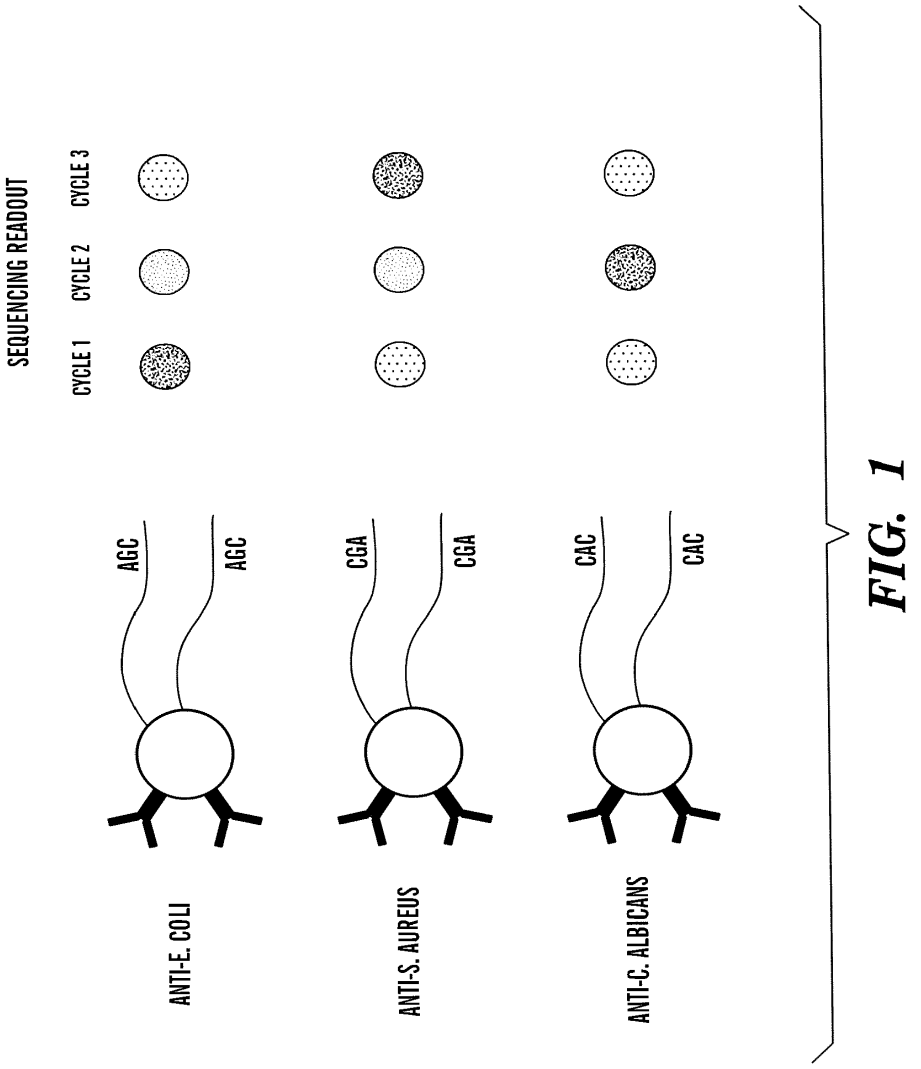


FIG. 1

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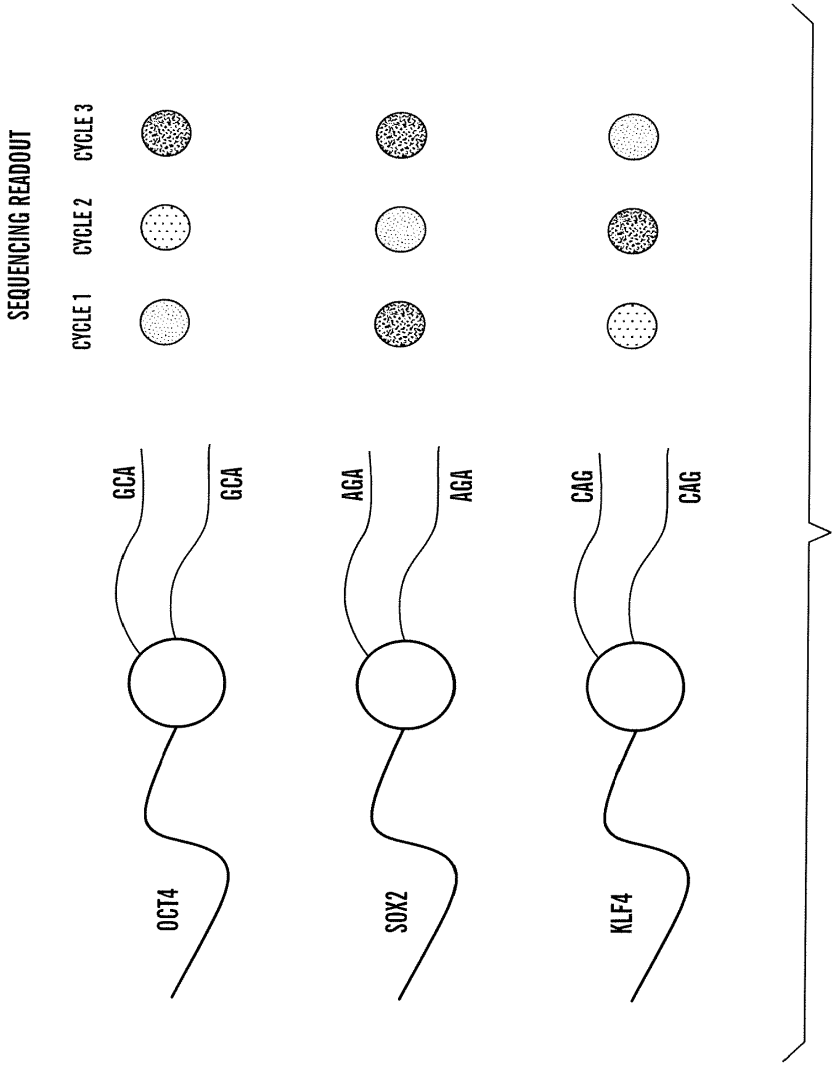


FIG. 2

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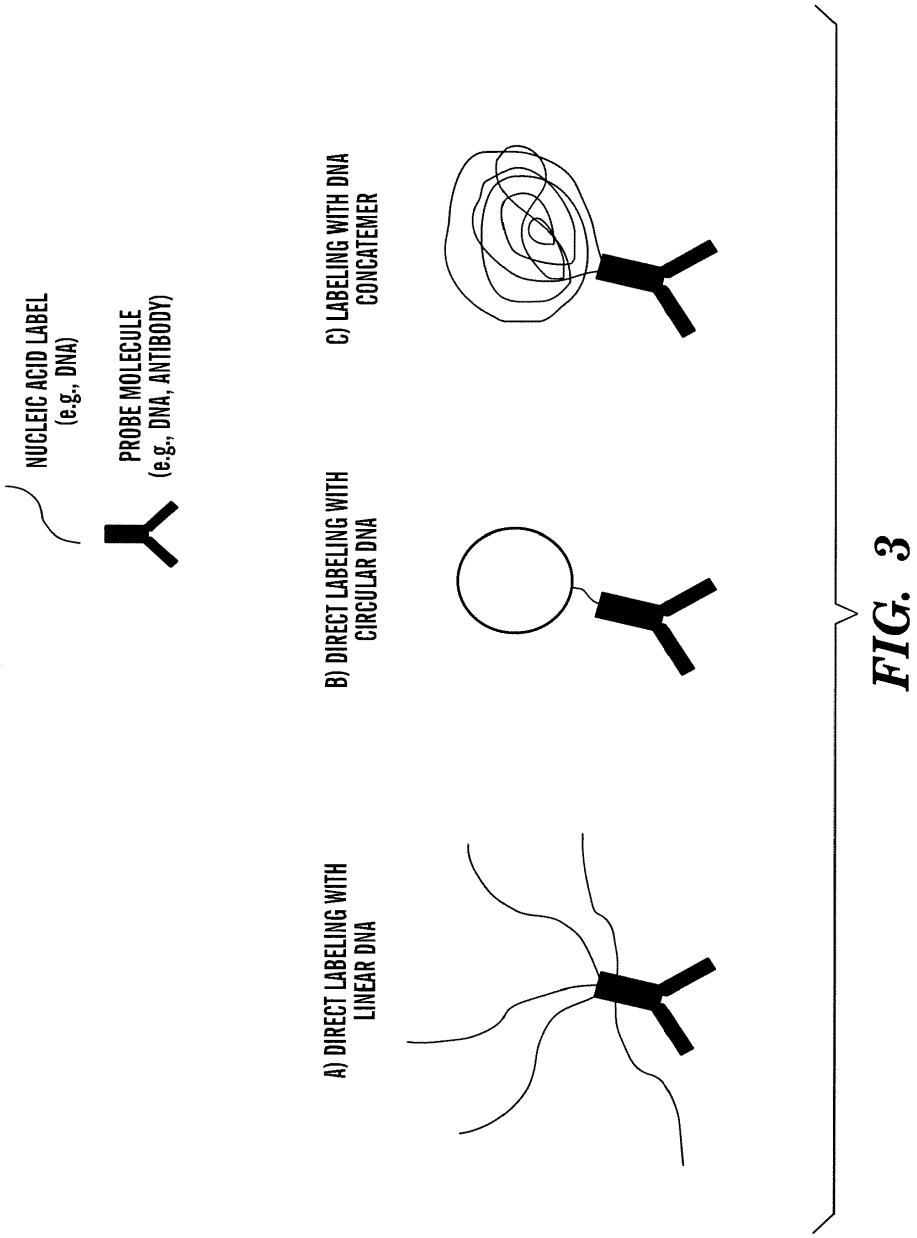


FIG. 3

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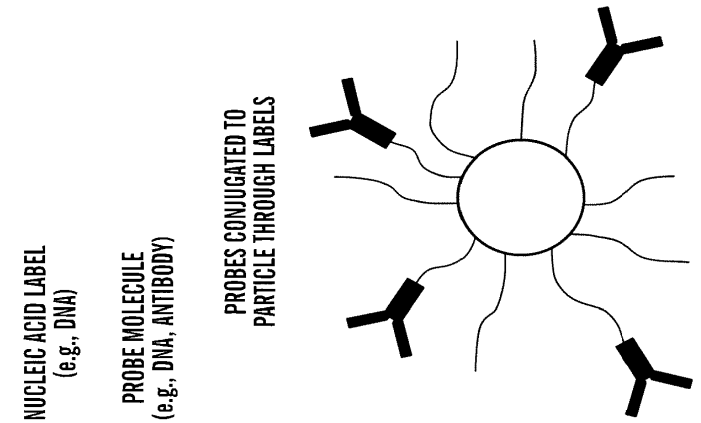


FIG. 4B

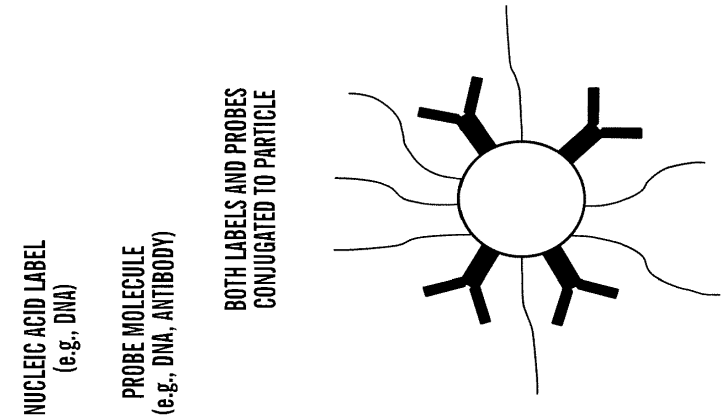


FIG. 4A

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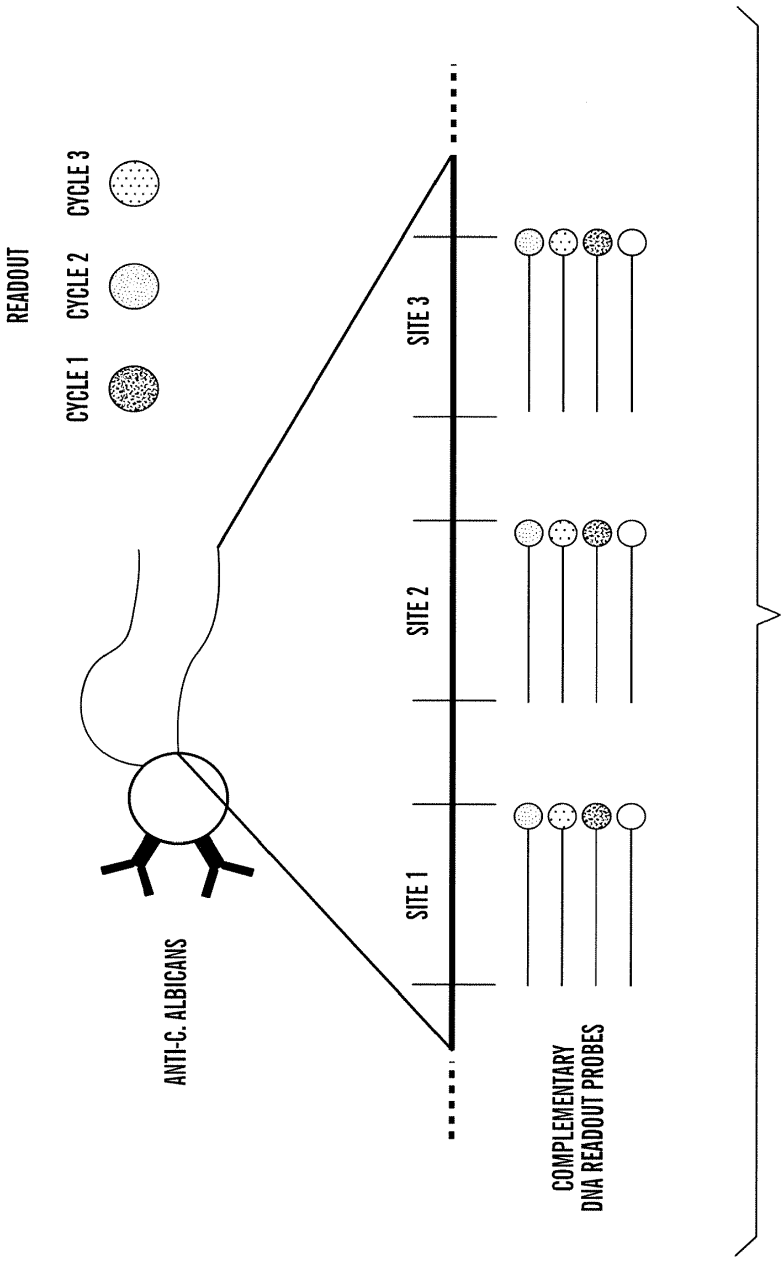


FIG. 5

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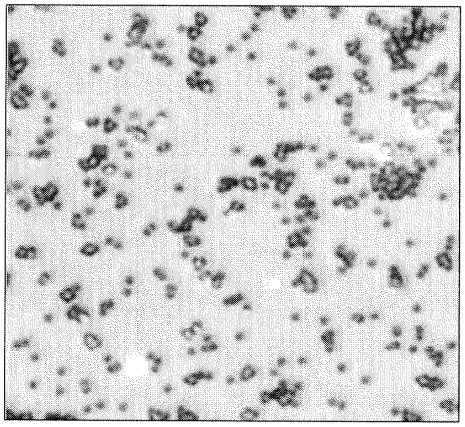


FIG. 6B

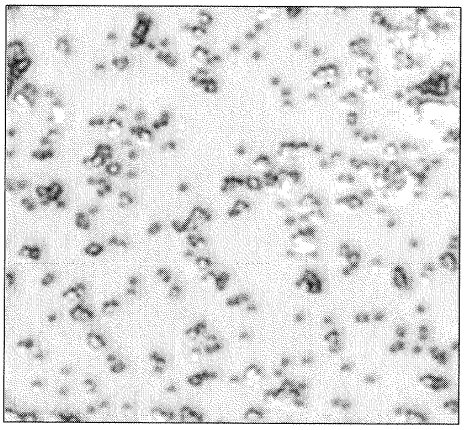


FIG. 6A

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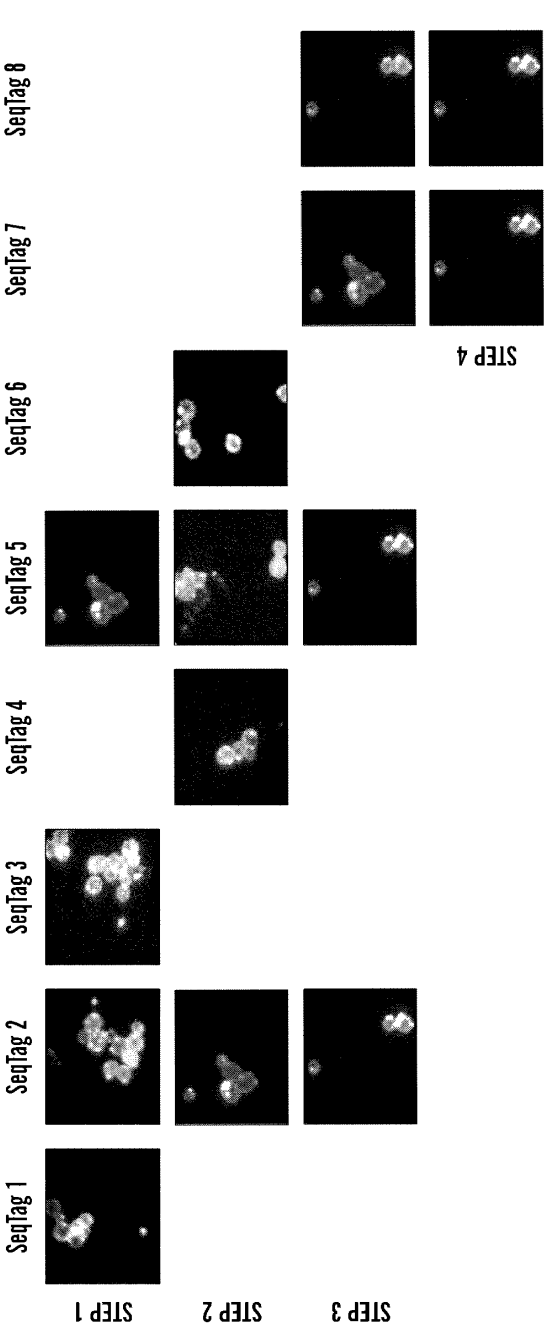


FIG. 7

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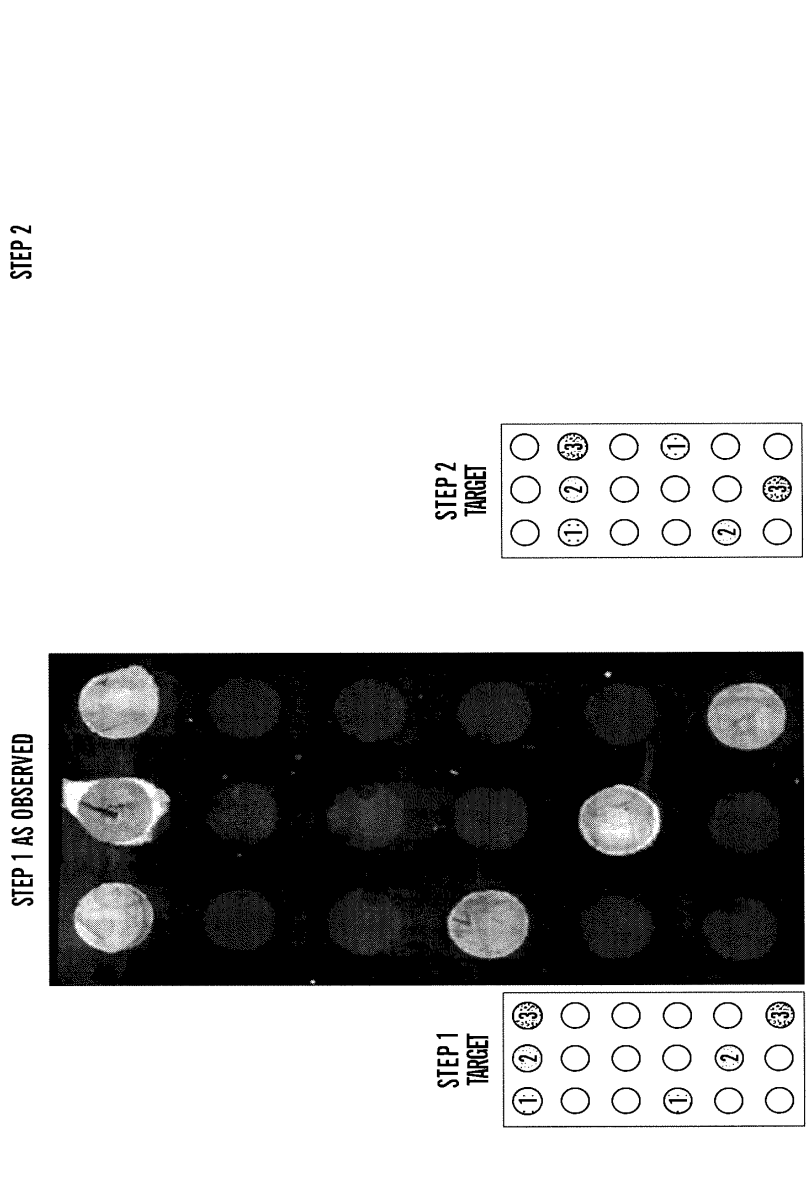


FIG. 8

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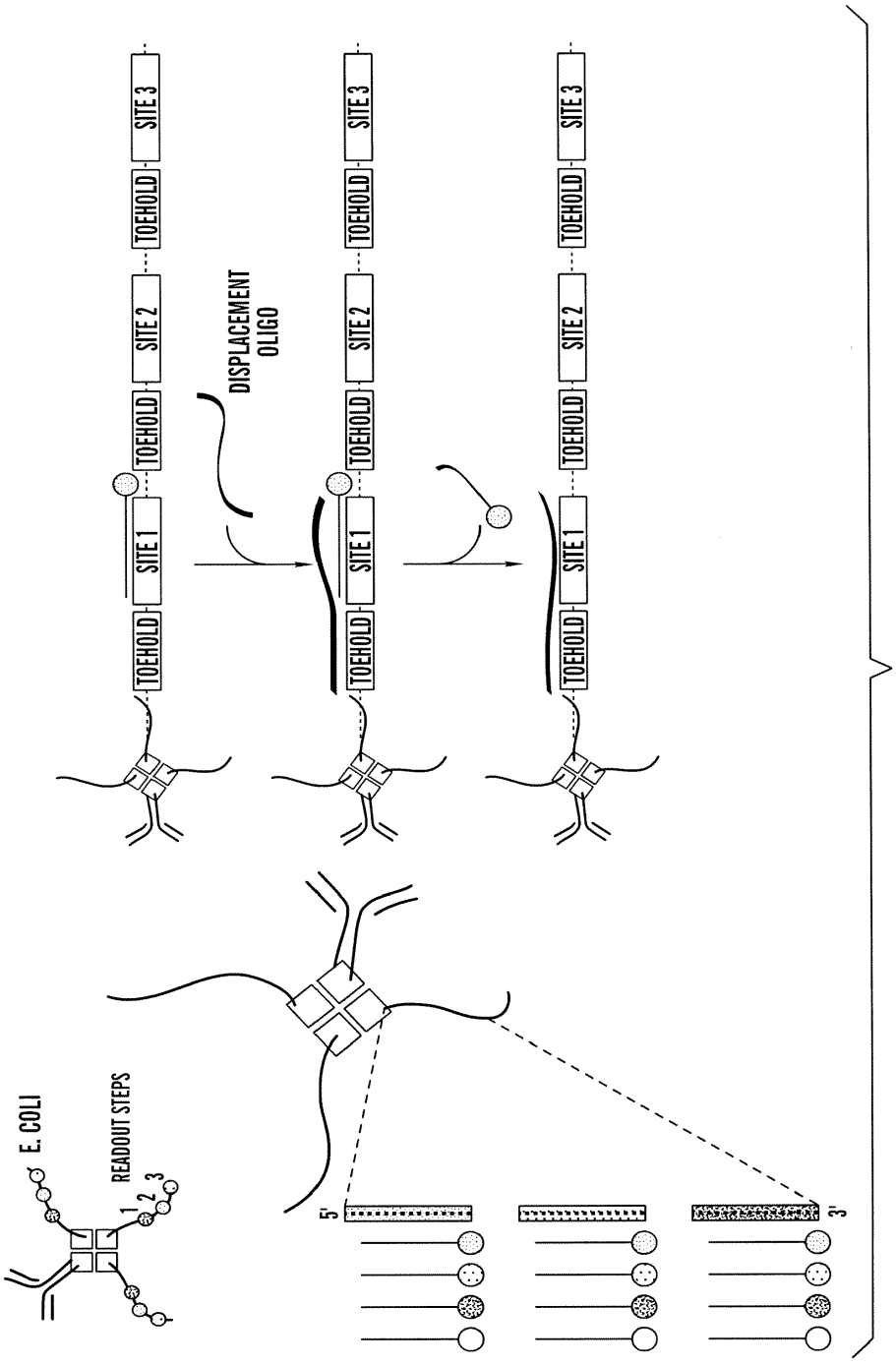


FIG. 9

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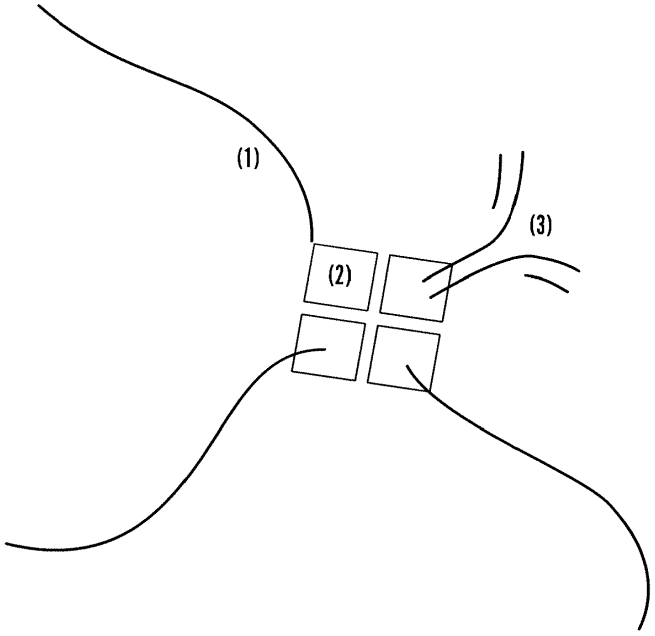


FIG. 10

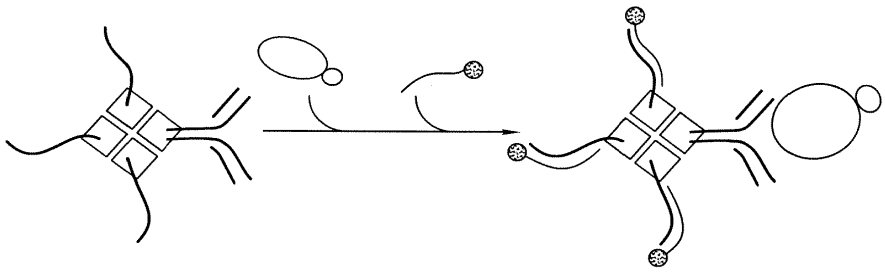


FIG. 11

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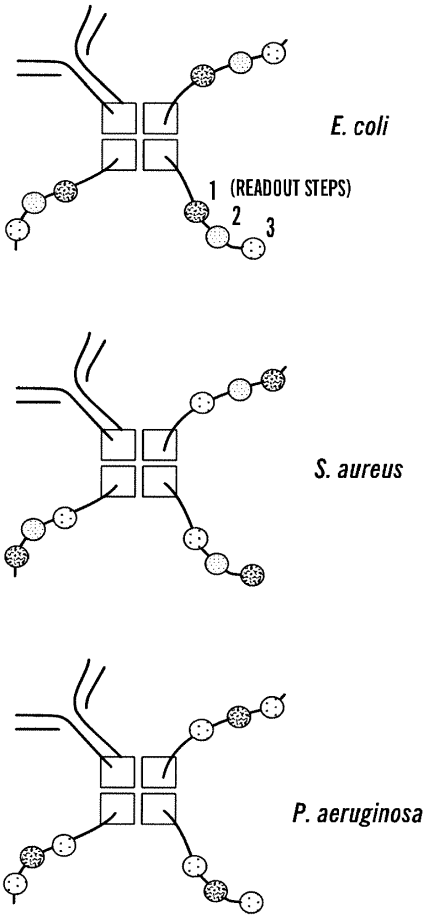


FIG. 12

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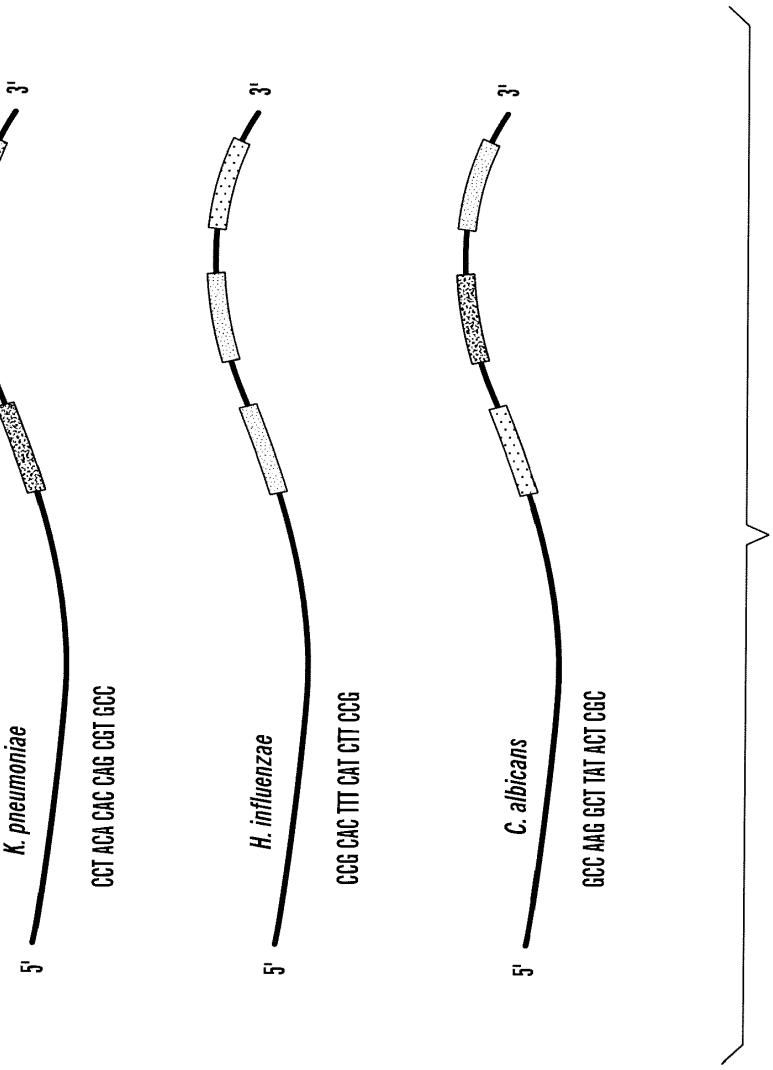


FIG. 13

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/071398

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i, C12N 15/11(2006.01)i, G01N 33/68(2006.01)i, G01N 33/532(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q 1/68; C07K 19/00; G01N 33/48; G01N 33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords:temporal, plural, detection, analyte, reagent, nucleic acid, label, conjugate, signal, optical

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011-0294135 A1 (CARLSON) 01 December 2011 See claims 1, 10, 13 and 24; paragraphs 0067, 0109, 0115 and 0119; and figure 1.	1-3,70-73
A	BRENNER et al., `Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays`, Nature Biotechnology, Vol. 18, pp. 630-634 (31 June 2000) See figure 2.	1-3,70-73
A	US 2009-0208965 A1 (TAFAS et al.) 20 August 2009 See the whole document.	1-3,70-73
A	KR 10-2008-0003402 A (MYCOSOL, INC.) 07 January 2008 See the whole document.	1-3,70-73
A	HAN et al., `Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules', Nature Biotechnology, Vol. 19, pp. 631-635 (31 July 2001) See the whole document.	1-3,70-73

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 April 2013 (05.04.2013)	**08 April 2013 (08.04.2013)**
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer KIM, Seung Beom Telephone No. 82-42-481-3371

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/071398

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: See extra sheet.
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See extra sheet.

3. ☒ Claims Nos.: See extra sheet.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

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(Contination of Box No.II)

2. Claims Nos.: 9, 12, 15, 23-24, 31, 36, 38, 40-41, 45, 47, 54-55, 59, 61, 75, 77, 82, 84, 92, 97, 99, 109 and 114

Claims 9, 12, 15, 23-24, 31, 36, 38, 40-41, 45, 47, 54-55, 59, 61, 75, 77, 82, 84, 92, 97, 99, 109 and 114 do not comply with PCT Article 6 because they are referring to multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. Claims Nos.: 4-8, 10-11, 13-14, 16-22, 25-30, 32-35, 37, 39, 42-44, 46, 48-53, 56-58, 60, 62-69, 74, 76, 78-81, 83, 85-91, 93-96, 98, 100-108, 110-113 and 115-117

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2012/071398

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011-0294135 A1	01.12.2011	EP 2185725 A1	19.05.2010
		EP 2185725 A4	12.01.2011
		WO 2009-018576 A1	05.02.2009
US 2009-0208965 A1	20.08.2009	AU 2007-329748 A1	12.06.2008
		CA 2667517 A1	12.06.2008
		CN 101754973 A	23.06.2010
		EP 2078030 A2	15.07.2009
		EP 2406390 A1	18.01.2012
		JP 2010-508028 A	18.03.2010
		KR 10-2009-0071667 A	01.07.2009
		US 2008-0182253 A1	31.07.2008
		US 2008-0213769 A1	04.09.2008
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		WO 2010-104533 A2	16.09.2010
KR 10-2008-0003402 A	07.01.2008	AU 2006-336403 A1	02.08.2007
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		WO 2007-056556 A2	18.05.2007
		WO 2007-086900 A2	02.08.2007

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Electronic Acknowledgement Receipt	
EFS ID:	26631663
Application Number:	14451876
International Application Number:	
Confirmation Number:	1065
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Customer Number:	21971
Filer:	Vernon A. Norviel/diane garcia
Filer Authorized By:	Vernon A. Norviel
Attorney Docket Number:	38075-716.301
Receipt Date:	12-AUG-2016
Filing Date:	05-AUG-2014
Time Stamp:	16:56:01
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		38075-716-301.pdf	259964 7388d934abf45bd9c26f94a8e2418f1b646822 94	yes	6

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	4	
	Information Disclosure Statement (IDS) Form (SB08)		5	6	
Warnings:					
Information:					
2	Foreign Reference	WO03002979.pdf	4856738 359ec52c129e85ee3ae5cea82bc5d60b1a45e2af	no	87
Warnings:					
Information:					
3	Foreign Reference	WO13096851.pdf	1380354 a806d492e7a50c63c580ccab6de2c543a8c9a7cd	no	128
Warnings:					
Information:					
4	Non Patent Literature	CHAPIN.pdf	346608 0016983f062aa0c88bf5c0db84af8a7be808b411	no	5
Warnings:					
Information:					
5	Non Patent Literature	EP137442612_EESR_03MAY2016.pdf	999929 0ac2cecd5a93467b0ad126e9cdc1fc2e697edbac	no	20
Warnings:					
Information:					
6	Non Patent Literature	HAN.pdf	911483 397ca2f2da7d227b4a78aa5cdf20b4e32e8a1f32	no	5
Warnings:					
Information:					
7	Non Patent Literature	KLOSTRANEC_ABST.pdf	107328 dcf5aa9dbabc85e6a94eede975783aef98716644	no	2
Warnings:					

Information:					
8	Non Patent Literature	LI.pdf	916601 55ea9e99b489f897778417f9b366168d582763c0	no	5
Warnings:					
Information:					
9	Non Patent Literature	LIN.pdf	696812 047035bc63d8e9bb8146d051fe50c34246452180	no	6
Warnings:					
Information:					
10	Non Patent Literature	XU.pdf	879531 7db945bcbca426c5d49917fd9b2f506677564e04	no	10
Warnings:					
Information:					
			Total Files Size (in bytes):	11355348	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	KARTALOV; Emil et al.	Group Art Unit:	1631
Serial Number:	14/451,876	Examiner:	NEGIN , RUSSELL SCOTT
Filing or 371 (c) Date:	2014-08-05	**CONFIRMATION NO:**	**1065**
Title:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

FILED ELECTRONICALLY ON: August 12, 2016

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

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A. ☒ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☒ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☐ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution on the merits, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☐ a fee of $180.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113 or (2) a notice of allowance under § 1.311, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $180.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

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E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 is NOT enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

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I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 23-2415 (Docket No. 38075-716.301).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: August 12, 2016

By: /Reena Bajpai/
Reena Bajpai, Reg. No. 69996

650 Page Mill Road
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Customer No. 21971

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 1065
Inventor(s): Kartalov, Emil P., et al.	Examiner: Russell Scott Negin
Application No.: 14/451,876	Art Unit: 1631
Filed: August 5, 2014	Customer No.: 21971
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO RESTRICTION REQUIREMENT

Dear Sir:

This communication is in response to the Restriction Requirement mailed on May 12, 2016 ("Restriction Requirement"). Reconsideration of the above-referenced application is respectfully requested in view of the following remarks.

Amendments to the Claims begin on page **2** of this paper.

Remarks begin on page **8** of this paper.

AMENDMENTS TO THE CLAIMS

1. **(Withdrawn)** An assay that is capable of unambiguously detecting the presence or absence of each of at least seven (7) analytes, in any combination of presence or absence, in a single sample volume without immobilization of said analytes, physical separation of said analytes, melting curve analysis, or mass spectrometry.
2. **(Withdrawn)** The assay of claim 1, wherein each of said analytes is encoded as a value of one component of a signal.
3. **(Canceled)**
4. **(Withdrawn)** The assay of claim 2, wherein each of said analytes is encoded as at least one first value in a first component of said signal and at least one second value in a second component of said signal.
5. **(Withdrawn)** The assay of claim 2, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

6.-8. **(Canceled)**

9. **(Withdrawn)** The assay of claim 4, wherein said first value is an intensity or range of intensities and said second value is a wavelength or range of wavelengths.

10.-12. **(Canceled)**

13. **(Withdrawn)** The assay of claim 2, wherein said signal is an electromagnetic signal.

14.-16. **(Canceled)**

17. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with reagents comprising hybridization probes.

18. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with a plurality of hybridization probes, comprising an identical fluorophore, wherein said plurality of hybridization probes are specific for different analytes.

19. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with a plurality of hybridization probes comprising different fluorophores, wherein said plurality of hybridization probes are specific for different analytes.

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20.-21. **(Canceled)**

22. **(Withdrawn)** The assay of claim 2, wherein said signal is generated during a polymerase chain reaction.

23.-26. **(Canceled)**

27. **(Withdrawn)** The assay of claim 4, further comprising constructing a diagram.

28. **(Withdrawn)** The assay of claim 27, wherein said diagram is constructed by plotting possible combinations of said first values and said second values for positive control samples.

29. **(Withdrawn)** The assay of claim 1, wherein at least one of said analytes comprises a polynucleotide.

30.-33. **(Canceled)**

34. **(Withdrawn)** The assay of claim 1, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.

35. **(Withdrawn)** The assay of claim 1, further comprising providing information concerning the presence or absence of said analyte to a health care professional, wherein the health care professional makes a clinical decision based on said information.

36. **(Canceled)**

37. **(Withdrawn)** The assay of claim 1, wherein at least one step of said assay is performed using a computer.

38. **(Withdrawn)** The assay of claim 37, wherein said computer is located on a remote server.

39. **(Withdrawn)** The assay of claim 37, wherein said computer is located on a thermal cycler.

40.-41. **(Canceled)**

42. **(Withdrawn)** The assay of claim 2, wherein said coding is innately non-degenerate.

43. **(Withdrawn)** The assay of claim 42, wherein said coding is designed to be non-degenerate by enumerating legitimate results that can be obtained from said encoding, identifying legitimate results that are degenerate, and eliminating at least one potential analyte code from

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said encoding to eliminate degeneracy.

44. **(Canceled)**

45. **(Withdrawn)** The assay of claim 1, wherein said assay is an end-point assay.

46. **(Canceled)**

47. **(Withdrawn)** The assay of claim 1, wherein said assay is a liquid phase assay.

48. **(Canceled)**

49. **(Currently Amended)** A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

    a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;
    b. providing a sample comprising, or potentially comprising, at least one of said analytes;
    c. contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;
    d. cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and
    e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme;
    wherein said method is capable of non-degenerately detecting the presence or absence of at least seven analytes in a single sample volume, in any combination of presence or absence.

50. **(Canceled)**

51. **(Original)** The method of claim 49, wherein said first value is an intensity or range of intensities.

52. **(Canceled)**

53. **(Previously Presented)** The method of claim 49, wherein said first value is incremented in said coding scheme to an amount greater than the sum of said preceding first values.

54. **(Canceled)**

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55. **(Currently Amended)** A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

a.[[f.]] encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

b.[[g.]] providing a sample comprising, or potentially comprising, at least one of said analytes;

c.[[h.]] contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present;

d.[[i.]] cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and

e.[[j.]] determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme,

wherein, when degeneracy is eliminated, said method is capable of ~~unambiguously~~ non-degenerately detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

56. **(Original)** The method of claim 55, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

57.-74. **(Canceled)**

75. **(New)** The method of claim 49, wherein each of said analytes is encoded as said first value of one component of said signal.

76. **(New)** The method of claim 75, wherein each of said analytes is encoded as at least one first value in said first component of said signal and at least one second value in a second

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component of said signal.

77. **(New)** The method of claim 75, wherein each of said analytes is encoded as said first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

78. **(New)** The method of claim 76, wherein said first value is an intensity or range of intensities and said second value is a wavelength or range of wavelengths.

79. **(New)** The method of claim 49, wherein said signal is an electromagnetic signal.

80. **(New)** The method of claim 49, wherein said detecting is performed with reagents comprising hybridization probes.

81. **(New)** The method of claim 49, wherein said detecting is performed with a plurality of hybridization probes, comprising an identical fluorophore, wherein said plurality of hybridization probes are specific for different analytes.

82. **(New)** The method of claim 49, wherein said detecting is performed with a plurality of hybridization probes comprising different fluorophores, wherein said plurality of hybridization probes are specific for different analytes.

83. **(New)** The method of claim 49, wherein said signal is generated during a polymerase chain reaction.

84. **(New)** The method of claim 49, further comprising constructing a diagram.

85. **(New)** The method of claim 84, wherein said diagram is constructed by plotting possible combinations of said first values and said second values for positive control samples.

86. **(New)** The method of claim 49, wherein at least one of said analytes comprises a polynucleotide.

87. **(New)** The method of claim 49, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.

88. **(New)** The method of claim 49, further comprising providing information concerning the presence or absence of said analyte to a health care professional, wherein the health care professional makes a clinical decision based on said information.

89. **(New)** The method of claim 49, wherein said encoding is innately non-degenerate.

90. **(New)** The method of claim 89, wherein said encoding is designed to be non-degenerate by enumerating legitimate results that can be obtained from said encoding, identifying legitimate

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results that are degenerate, and eliminating at least one potential analyte code from said encoding to eliminate degeneracy.

91. **(New)** The method of claim 49, wherein said method does not comprise immobilization of said analytes.

92. **(New)** The method of claim 55, wherein said method does not comprise immobilization of said analytes.

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REMARKS

Claims 1-2, 4-5, 9, 13, 17-19, 22, 27-29, 34-35, 37-39, 42-43, 45, 47, 49, 51, 53, 55, and 56 were pending prior to the entry of the above-mentioned amendments. Claims 1-2, 4-5, 9, 13, 17-19, 22, 27-29, 34-35, 37-39, 42-43, 45, and 47 are withdrawn. Claims 3, 6-8, 10-12, 14-16, 20-21, 23-26, 30-33, 36, 40-41, 44, 46, 48, 50, 52, 54, and 57-74 are canceled.

Claims 75-92 are new. Support for new claims 75-92 can be found at least in the originally filed claims and paragraphs [0003]-[0023], [00119], and [00179] of the filed specification.

Claims 49 and 55 are amended. Support for amended claims 49 and 55 can be found at least in paragraphs [0003], [0023], [0059], and [0084]-[0085] of the filed specification. Claim 55 has also been amended to correct typographical errors.

No new matter has been added. Accordingly, claims 49, 51, 53, 55-56, and 75-92 are currently pending.

Reconsideration of this application is respectfully requested in light of the following remarks.

Election/Restrictions

The Office has required an election of an invention under 35 U.S.C. § 121 to one of the following species.

I. Claims 1-2, 4-5, 9, 13, 17-19, 22, 27-29, 34-35, 37-39, 42-43, 45 and 47 drawn to an assay that does not use immobilization of analytes, separation of analytes, melting curve analysis, or mass spectrometry.

II. Claims 49, 51, 53, and 55-56 drawn to using cumulative measurements to determine the presence or absence of each analyte in a plurality of analytes.

Applicant elects, without traverse, invention II, encompassing claims 49, 51, 53, and 55-56 and new claims 75-92.

The Office is also requiring an election of a single patentably distinct species from each of the following sets of species:

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I. Species A: the plurality of fluorescent probes comprise an identical fluorophore (claim 18)

II. Species B: the plurality of fluorescent probes comprise different fluorophores (claim 19)

Applicant elects, without traverse, Species A, encompassing claims 18, 49, 51, 53, and 55-56 and new claims 75-92.

Applicant reserves the right, pursuant to 35 U.S.C. § 121, to file one or more divisional applications directed to the non-elected inventions during the pendency of the present application.

Applicant respectfully submits that upon the allowance of a generic claim, Applicant shall be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141.

***

It shall be understood herein that any instance in which Applicant has addressed certain comments set forth by the Office shall not be construed as a concession to other comments or arguments advanced by the Office. Any circumstance in which Applicant has amended or canceled a claim also does not mean that Applicant concedes to the arguments or positions advanced by the Office with respect to that claim or other claims pending herein.

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CONCLUSION

Applicant submits that this paper fully addresses the Office Action mailed May 12, 2016. Should the Examiner have any questions, the Examiner is encouraged to contact the undersigned.

The Commissioner is hereby authorized to charge any additional fees or credit any overpayment in connection with this paper to Deposit Account No. 23-2415, Attorney Docket No. 38075-716.301.

Respectfully submitted,

Dated: November 3, 2016

By: /Andrew L. Chang/
Andrew L. Chang
Reg. No. 75,298

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 2126

Electronic Patent Application Fee Transmittal

Application Number:	14451876
Filing Date:	05-Aug-2014
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Filer:	Jacqueline D. Stroncek/Cecilia Hui/ANCH
Attorney Docket Number:	38075-716.301

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	2202	18	40	720
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Extension - 4 months with $0 paid	2254	1	1100	1100
Miscellaneous:				
		Total in USD ($)		**1820**

Electronic Acknowledgement Receipt	
EFS ID:	27415690
Application Number:	14451876
International Application Number:	
Confirmation Number:	1065
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Customer Number:	21971
Filer:	Jacqueline D. Stroncek/Cecilia Hui/ANCH
Filer Authorized By:	Jacqueline D. Stroncek
Attorney Docket Number:	38075-716.301
Receipt Date:	03-NOV-2016
Filing Date:	05-AUG-2014
Time Stamp:	19:43:40
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1820
RAM confirmation Number	110416INTEFSW00005811232415
Deposit Account	232415
Authorized User	Andrew Chang
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		38075_716301_Response_RR.pdf	173803 2bc1d60657fd0de3c0e9674e4d064029cf5ddb57	yes	10
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Response to Election / Restriction Filed		1	1	
	Claims		2	7	
	Applicant Arguments/Remarks Made in an Amendment		8	10	
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	32788 40688927009c8f915f9531fb7ac932880a5416f7	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	206591		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/451,876	Filing Date 08/05/2014	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	11/03/2016						
	Total (37 CFR 1.16(i))	* 45	Minus	** 27	= 18	X $40 =	720
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	X $210 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	**720**

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

SLIE
BRENDA L. GRAY

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Document code: WFEE

United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/451,876	08/05/2014	Emil Kartalov	38075-716.301	1065

21971 7590 11/23/2016

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1631	

NOTIFICATION DATE	DELIVERY MODE
11/23/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

Office Action Summary	Application No. 14/451,876	Applicant(s) KARTALOV ET AL.	
	Examiner Russell S. Negin	Art Unit 1631	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *3 November 2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *See Continuation Sheet* is/are pending in the application.

5a) Of the above claim(s) *See Continuation Sheet* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *49,51,53,55,56,75-81 and 83-92* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *5 August 2014* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *See Continuation Sheet*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

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Continuation of Disposition of Claims: Claims pending in the application are 1,2,4,5,9,13,17-19,27-29,34,35,37-39,42,43,45,47,49,51,53,55,56 and 75-92.
Continuation of Disposition of Claims: Claims withdrawn from consideration are 1,2,4,5,9,13,17-19,27-29,34,35,37-39,42,43,45,47, and 82.

Continuation of Attachment(s) 2). Information Disclosure Statement(s) (PTO/SB/08), Paper No(s)/Mail Date :12/2/2014; 1/25/2016; 8/12/2016.

DETAILED ACTION

Comments

The present application is being examined under the pre-AIA first to invent provisions. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

Election/Restrictions

Applicant's election without traverse of Group II and Species A in the reply filed on 3 November 2016 is acknowledged.

Claims 1-2, 4-5, 9, 13, 17-19, 27-29, 34-35, 37-39, 42-43, 45, 47, and 82 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected Group or Species, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on 3 November 2016.

Claims 1-2, 4-5, 9, 13, 17-19, 27-29, 34-35, 37-39, 42-43, 45, 47, 49, 51, 53, 55-56, and 75-92 are pending in the instant application.

Claims 49, 51, 53, 55-56, 75-81, and 83-92 are examined in the instant Office action.

Information Disclosure Statements

The IDSs filed on 12/2/2014, 1/25/2016, and 8/12/2016 have been considered.

Claim Rejections - 35 USC § 112(b) - Indefiniteness

The following is a quotation of 35 U.S.C. 112(b):
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 89 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

The term "innately" in claim 89 is a relative term which renders the claim indefinite. The term "innately" is not defined by the claim, the specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention. It is unclear as to the metes and bounds of what makes encoding non-degenerate versus innately non-degenerate. For the purpose of examination, it is interpreted that any non-degenerate encoding is innately non-degenerate.

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 49, 51, 53, 55-56, 75-81, and 83-92 are rejected under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more. According to Step 1 of the 2014 Interim Subject Matter Eligibility Guidelines, claim(s) 49, 51, 53, 55-56, 75-81, and 83-92 is/are directed to methods. According to Step 2A of the 2014 Interim Subject Matter Eligibility Guidelines, claim(s) 49, 51, 53, 55-56, 75-81, and 83-92 is/are directed to the abstract idea of encoding signals in a manner that reduces or eliminates degeneracy. The signal processing is a manner of mathematical manipulation analogous to the Arrhenius equation of *Diamond v. Diehr*, 450 U.S. 175 (1981), which was also found to be abstract. The claim(s) does/do not include additional elements that are sufficient to amount to significantly more than the judicial exception (i.e. Step 2B of the Guidelines) because providing samples, performing assays, and determine presence/absence patterns of analytes is routine and conventional in the prior art. This rejection has been made in view of the U.S. Supreme Court Decision in *Alice Corporation Pty. Ltd. V. CLS Bank International, et al*.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –

> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claim(s) 49, 51, 53, 55-56, 75-81, 84-85, and 88-92 is/are rejected under pre-AIA 35 U.S.C. 102(b) as being anticipated by Nie et al. [US PGPUB 2003/0148544 A1; on IDS].

Claim 49 is drawn to a method of detecting the presence or absence of each analyte of a plurality of analytes. The method comprises encoding each of the analytes as a first value of a signal, thereby generating a coding scheme, wherein each of the analytes is represented in the coding scheme by the first value, wherein the encoding is performed in a manner that reduces or eliminates degeneracy. The method comprises providing a sample comprising at least one of said analytes. The method comprises contacting the sample with analyte-specific reagents that generate the first value, as represented in the coding scheme, when each of the analytes is present. The method comprises cumulatively measuring the first values of the signal within the sample, thereby providing a cumulative measurement. The method comprises determining whether each of the analytes is present or absent based on the cumulative measurement and the coding scheme. The method is capable of non-degenerately detecting the presence or absence of at least seven analytes in a single sample volume, in any combination of presence or absence.

The capability of “non-degenerately detecting the presence or absence of at least seven analytes in a single sample volume, in any combination of presence or absence” is interpreted to be an intended use that does not differentiate the recitations of the claims from the prior art.

Claim 55 is drawn to similar subject matter as claim 49, the additional limitation that each of the analytes corresponds to both a first value and a second value.

The document of Nie et al. studies methods of preparing multicolor quantum dot tagged beads [title]. The cover figure of Nie et al. teaches that different color quantum dots within microbeads. Each type of quantum dot emits light at a different wavelength. The histograms in the cover figure of Nie et al. illustrates how the quantum dot composition within each microbead encodes a non-degenerate spectral signature in the form of intensity versus wavelength histogram. In the cover figure of Nie et al., the quantum dots are the analytes in contact with the microbead to generate the optical wavelength used in the coding scheme. The height of each bar in each histogram represents the cumulative measurement of all of the quantum dots of a particular type within each microbead. The existence of a bar in each histogram indicates presence of a specific analyte emitting at a particular wavelength. The absence of a bar in each histogram indicates absence of a specific analyte emitting at a particular wavelength. It is interpreted that the first value corresponding to the analyte in the microbead is intensity, and the second value is the wavelength.

With regard to claims 51 and 75, the first value represents the fluorescent intensity value in the histograms of the cover figure of Nie et al. Each type of analyte in each microbead is encoded as a specific intensity value.

With regard to claim 53, the cover figure of Nie et al. comprises incrementing the first value in an amount greater than the sum of the preceding values.

With regard to claims 76-78, in the cover figure of Nie et al., it is interpreted that the first values corresponding to the analyte in the microbead are intensities, and the second values are the wavelengths.

With regard to claim 79, it is interpreted that the photons emitted from the quantum dots in the microbeads in the cover figure of Nie et al. to result in colored light comprises electromagnetic signaling.

With regard to claims 80-81, Figure 5 of Nie et al. teaches the use of hybridization probes to generate the probes wherein each combination of analytes within each microbead corresponds to a different hybridization probe. Each microbead in Figure 5 of Nie et al. comprises the three quantum dot analyte fluorophores.

With regard to claims 84-85, the cover figure of Nie et al. illustrates a diagram plotting possible combinations of first and second values for positive control samples.

With regard to claim 88, paragraph 4 of Nie et al. teaches an application of the optical signatures of Nie et al. is in clinical diagnostics.

With regard to claims 89-90, Figure 1 and 5 and Nie et al. illustrate non-degenerate, legitimate results. No results are eliminates because the data in Figures 1 and 5 of Nie et al. are non-degenerate.

With regard to claims 91-92, the analytes in Figures 1 and 5 of Nie et al. are in microbeads that are freely moving and not immobilized.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

35 U.S.C. 103 Rejection #1:

Claim 86 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Nie et al. as applied to claims 49, 51, 53, 55-56, 75-81, 84-85, and 88-92 above in further view of Craig et al. [Nucleic Acids Research, volume 18, 1990, pages 2653-2660; on IDS].

Claim 86 is further limiting wherein at least one of the analytes comprises a polynucleotide.

Nie et al. teaches optical encoding of analytes in microbeads as a result of intensities and wavelengths of optical signals emitted from the analytes, as discussed above.

Nie et al. does not teach that the analytes comprise polynucleotides.

The document of Craig et al. studies ordering of cosmid clones covering Herpes simplex virus type I (HSV-I) genome for use in fingerprinting. Figure 1 of Craig et al. lists the 22 polynucleotide analytes used to encode presence/absence patterns for the purpose of differentiating genomes by hybridization.

It would have been obvious for someone of ordinary skill in the art at the time of the invention to modify the optical encoding of Nie et al. by use of the hybridization encoding of Craig et al. because it is obvious to combine known elements in the prior art to yield a predictable result. In this instance, hybridization encoding is an alternative to optical encoding. There would have been a reasonable expectation of success in combining Nie et al. and Craig et al. because both studies are analogously applicable to using presence/absence patterns as signatures for analyte combinations.

35 U.S.C. 103 Rejection #2:

Claim 83 and 87 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Nie et al. in view of Craig et al. as applied to claims 49, 51, 53, 55-56, 75-81, 84-86, and 88-92 above in further view of Orpana et al.[WO 2010/128206 A1].

Claim 83 is further limiting wherein the signal is generated by PCR.

Claim 87 is further limiting comprising transiting information via a computer network.

Nie et al. and Craig et al. make obvious using presence/absence patterns of biomolecules in coding schemes, as discussed above.

Nie et al. and Craig et al. do not teach PCR or computer automation.

Orpana et al. studies a method, instrument, and computer program product for quantification of PCR products [title].

It would have been obvious for someone of ordinary skill in the art at the time of the invention to modify the optical encoding of Nie et al. and the hybridization encoding of Craig et al. by use of the computerized PCR of Orpana et al. wherein the motivation would have been that computer automate the analysis and PCR facilitates understanding of the experiment by amplifying the results of the experiment [title and abstract of Orpana et al.].

E-mail Communications Authorization

Per updated USPTO Internet usage policies, Applicant and/or applicant's representative is encouraged to authorize the USPTO examiner to discuss any subject

matter concerning the above application via Internet e-mail communications. See MPEP 502.03. To approve such communications, Applicant must provide written authorization for e-mail communication by submitting the following statement via EFS-Web (using PTO/SB/439) or Central Fax (571-273-8300):

> Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned and practitioners in accordance with 37 CFR 1.33 and 37 CFR 1.34 concerning any subject matter of this application by video conferencing, instant messaging, or electronic mail. I understand that a copy of these communications will be made of record in the application file.

Written authorizations submitted to the Examiner via e-mail are **NOT** proper. Written authorizations must be submitted **via EFS-Web** (using PTO/SB/439) **or Central Fax** (571-273-8300). A paper copy of e-mail correspondence will be placed in the patent application when appropriate. E-mails from the USPTO are for the sole use of the intended recipient, and may contain information subject to the confidentiality requirement set forth in 35 USC § 122. See also MPEP 502.03.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 10:30 am to 5 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Peter Paras, Supervisory Patent Examiner, can be reached at (571) 272-4517.

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/Russell S. Negin/
Primary Examiner, Art Unit 1631
17 November 2016

Notice of References Cited	Application/Control No. 14/451,876	Applicant(s)/Patent Under Reexamination KARTALOV ET AL.	
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*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
	A	US-				
	B	US-				
	C	US-				
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(54) **Title**: METHOD, INSTRUMENT AND COMPUTER PROGRAM PRODUCT FOR QUANTIFICATION OF PCR PRODUCTS

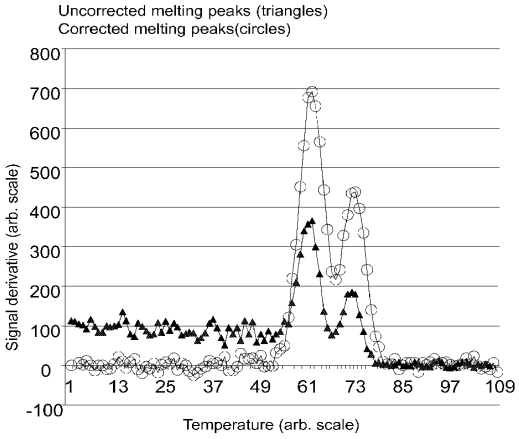


Fig. 2

(57) **Abstract**: This document relates to melting curve analysis of nucleic acids. The method according to first aspect of the invention comprises analyzing a nucleic acid melting curve measured from a sample, the melting curve comprising a sum signal of at least two nucleic acid melt signals and a background signal as a function of temperature. The method further comprises optimizing at least one constant in a temperature-dependent exponential correction function so as to minimize the variation of the nucleic acid melting curve at a temperature region where the target nucleic acids in the sample remain essentially double stranded, and generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal by applying said exponential correction function over the region of the measured melting curve where the strands of the nucleic acids dissociate. According to further aspects, the invention relates to a curve fitting algorithm for precise estimation of melting peak areas and a mathematical transformation for linearization of calibration curve data to enhance the linear measuring range of a competitive PCR assay. The invention provides a powerful tool for analyzing PCR-amplified sample containing two or more different nucleic acids having similar but distinguishable melting temperature.

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Method, instrument and computer program product for quantification of PCR products

FIELD OF THE INVENTION

The present invention relates to nucleic acid melting curve analysis. More specifically the embodiments of the present invention relate to the methods and systems for removing background signals and extracting specific signals in melting profiles of double stranded nucleic acids.

Melting curve analysis is typically performed in conjunction with polymerase chain reaction (PCR) for analysis of amplification products. The present invention thus also relates to instruments and software for melting curve analysis and real-time and competitive PCR techniques.

BACKGROUND OF THE INVENTION

Melting curve analysis is based on measurement of the temperature-dependent dissociation of double stranded nucleic acids, typically DNA, in a solution. The energy required for dissociation of the hydrogen bonds between the two strands is dependent on the length and base composition of the double stranded DNA molecules and on the complementarity of the opposing strands. The dissociation of the strands can be monitored using probes that change their fluorescent properties upon binding to nucleic acids, or fluorescent dyes that fluoresce when bound to double-stranded DNA but cease to fluoresce when DNA dissociates into single-stranded molecules. Thus, the amount of double stranded DNA present in a sample can be measured by optically monitoring the amount of fluorescence while increasing the temperature in a sample.

If the sample contains multiple groups of double stranded DNA molecules of different length or with a differing nucleotide composition, the result of the measurement will

comprise a sum of temperature-dependent fluorescent signals that are specific for each of the analyzed groups of DNA molecules. Thus, when measuring the PCR amplicons in a sample subsequent to PCR amplification of two or multiple templates, specific temperature-dependent fluorescent signals can be obtained for each of the specific PCR amplicons present in the sample.

In addition there is typically a decrease in fluorescent signal with an increasing temperature that occurs regardless of the temperature-specific dissociation of double stranded DNA molecules. This decrease in fluorescent signal can typically be observed over the whole temperature range, especially at temperatures where the DNA molecules in the sample remain essentially double stranded. This background signal is dependent on the amount of double stranded DNA present in the sample and it decreases exponentially as the temperature of the sample increases. The measured signal thus, comprises the specific decrease in fluorescent signal obtained from dissociation of double stranded DNA in the sample and of a temperature dependent exponentially decreasing background signal.

Real-time PCR involves amplification of cDNA or DNA templates and quantitative measurement of the amount of amplified DNA amplicons. In this technique the accumulation of PCR product in the reaction is monitored during each cycle of PCR amplification. The amount of template DNA present in a sample can be determined by comparing accumulation of reaction products in a sample to accumulation of reaction products in known standards. Additionally reaction efficiencies can be determined based on accumulation kinetics, but accuracy of such calculation is typically compromised by limited data points.Competitive PCR provides an alternative approach to control the variations in amplification efficiency between separate reaction vials. In this technique a known amount of a reference DNA template that differs in length or nucleotide composition from the wild type cDNA or DNA template, is included in the reaction and co-amplified with the wild type template in the same reaction vial. The relative amounts of wild type and reference PCR amplicons are determined subsequently to amplification. As, the amplification reaction efficiency influences both wild type and reference

templates equally, the amount of wild type template originally present in the sample can be determined from the relative amounts of wild type and reference PCR amplicons. In real-time PCR, quantitative measurement is performed during amplification, whereas in competitive PCR it is typically performed after completion of the amplification reaction.

Diagnostic tests based on RNA-expression analysis are faced with several technical challenges. A specific challenge has been the analysis of formalin-fixed paraffin-embedded (FFPE) tissue samples, in which the RNA molecules have been degraded into shorter fragments. Extraction and amplification of these short RNA fragments is technically demanding and the obtained signals are typically low. Genome-controlled RT-PCR has been used successfully for this purpose (*Stenman et al.*, *Clinical Chemistry 52:11 1988–1996 (2006)*). This technique is based on competitive PCR and enables amplification and quantitative measurement of short cDNA fragments, making it suitable for analysis of mRNA expression in FFPE samples. After competitive PCR amplification, the relative amounts of PCR amplicons originating from wild type cDNA and reference DNA templates are measured using melting curve analysis. In this technique the PCR amplicons generated from the wild type cDNA and reference DNA templates differ in nucleotide composition and differential detection is based on slight differences in the melting temperature. There are several specific benefits of this technique. Using genome-controlled RT-PCR it is possible to amplify exceptionally short cDNA fragments generated from mRNA of virtually any gene, as differentiation between expressed and genomic sequences is not dependent on intronic sequences. This is essential when analyzing FFPE samples. Co-amplification of a reference DNA template in every reaction regardless of the amount of wild type cDNA template present increases the reproducibility of the assay. The detection limit and imprecision of the assay can be controlled by varying the amount of the reference template, which is essential for diagnostic applications. Also, carryover of genomic DNA from the RNA extraction step does not cause false positive result following amplification. The requirements on the quantitative properties of the melting curve analysis in this type of assay are exceptionally high, as the relative levels of PCR products with close-to-equal melting temperatures are precisely quantified over a measuring range spanning several decades.

An instrument for rapid PCR amplification and detection of PCR products based on melting temperature is described in WO 1997/046712. The principle of melting curve analysis based on fluorescence measurements is extensively described in publications WO 2000/066777, WO 2004/038038 and WO 2006/121423. Extraction of qualitative and quantitative data from melting curve spectra typically involves measuring the fluorescent signal as a function of temperature to produce a raw melting curve. By calculating the rate of the change in signal as a function of temperature, a first derivative curve that displays dissociation of specific double stranded DNAs as specific "melting peaks", can be generated. A problem of this type of analysis is, however, the background signal, which affects the size and baseline of the separate melting peaks in the spectrum and complicates quantitative analysis especially when multiple peaks are present in the same spectra. In publication WO 2007/035806, an improvement of the analytical procedure is described in which the background signal is subtracted from the nucleic acid sample signal. In this technique an exponential algorithm is derived by interpolation from two separate slope values taken from points before and/or after the nucleic acid melting transition. This algorithm is then used to generate a corrected melting curve by subtracting the background from the raw melting curve data or from the first derivative melting curve spectra. This technique has been used primarily for genotyping applications, in which mutations in one or both alleles of a gene are detected. In these applications melting curve analysis has been used to detect dissociation of target-specific unlabelled probes from amplified DNA. Alternatively, whole amplicon melting curve analysis has been performed. In these types of applications, it is essential that the resolution of the assay is sufficient for separate detection of melting peaks occurring in the same spectrum.

Prior techniques for correcting the measured melting curve and for analyzing the melting peak areas, including those described in the abovementioned publications, are not capable of satisfactorily distinguishing between melting peaks which are situated very close to each other. In particular, improvements are needed for techniques for precise

quantification of the relative amounts of nucleic acids in the sample, in particular in RNA-expression analyses.

SUMMARY OF THE INVENTION

The aim of the present invention is to provide new techniques for accurate analysis of melting curve spectra. In particular, the aim of the present invention is to provide an improved method for relative quantification of two or more specific melting peaks present in a melting curve spectrum obtained from amplified DNA.

A further aim of the present invention is to improve instruments and software analysis for amplification and quantitative measurement of nucleic acids.

The invention has its specific focus on achieving techniques for accurate quantification of double-stranded DNA molecules, the length of which varies from 1000 to 30, in particular from 500 to 30 nucleotides. Specific advantages are obtained in studies involving molecules whose length is 100 or less, in particular 70 to 30 nucleotides.

The first aspect of the present invention is a signal-dependent method of transformation of raw melting curve data comprising the sum of the background signal and nucleic acid melt signal in order to render the data in an optimal form for final analysis. This is achieved by

- optimizing one or more constants in a temperature-dependent exponential correction function to render the decrease in the sum signal intensity close to zero at a temperature region of the raw melting curve where the target nucleic acids in the sample remain essentially double stranded, and
- generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal using said correction function, in particular by multiplying the entire raw melting curve or its derivative, or a specific area of interest thereof, with said correction function. The absolute amount of correction is thus dependent on the fluorescent signal value at each given temperature point.

According to a preferred embodiment, the corrected melting curve is obtained directly by multiplying the measured melting curve by a correction function which is exponentially dependent on the temperature. That is, the correction function includes at least one term having an exponential behavior with respect to temperature. Thus, separate computation or subtraction of the background signal from the measured signal is not required or carried out. The corrected raw melting curve comprises the fluorescent signal related to the specific dissociation of the target nucleic acids in the sample.

According to one embodiment, the correction function $C(T)$ includes a correction term which is proportional to K^{BT}, where K is an exponential base, T is temperature and B is a scaling factor. In particular, the correction function can be of the mathematical form given in the detailed description below. The corrected melting curve $F_{corr}(T)$ is obtained as the product $C(T)F_{meas}(T)$, where F_{meas} is the measured melting curve.

According to one embodiment, the measured melting curve is pre-processed before applying the correction function to it in order to correct a potential offset in the general level of the curve or the correction function itself includes one or more factors correcting such offset (thus, for simplicity, the terms "melting curve" and F_{meas} can refer either to original or offset-corrected curve). This offset correction renders the area of the melting curve after the melting transition to zero or close to zero. Offset correction compensates for any systematic measurement errors and/or systematic (constant) fluorescence background that may be present.

Determination of the base K can be carried out by normalization of the melting curve at a region where no dissociation of target nucleic acids occurs. This can in practice be implemented by optimizing the correction factor (K), so as to render the change in the background signal close to zero over the non-dissociating region. For example, minimum sum or least squares method can be utilized for the optimization of K. After that, the K-based correction function dependent on temperature (or temperature difference with

respect to a predefined starting point) at each point is applied to all points of the melting curve or a specified portion thereof.

For obtaining a melting peak curve, the derivative, typically a negative derivative, of the corrected melting curve is calculated. It is appreciated by a person skilled in the art that an equivalent to the above-described correction method is a method where a corresponding correction is carried out to an already derived curve. The derivative of a product rule may be applied for obtaining a correction function suitable for this case. That is, the order of steps is not critical.

The above-described approach provided significant advantages over prior art. Exponential correction function as a multiplier of the measured melting curve produces a background-corrected melting curve having a steep melting region, the derivation of which further produces a melting peak curve where closely-situated peaks are narrow and well distinguishable. In addition, the correction takes into account that the measured signal level per molecule at a higher temperature is lower than at a lower temperature. As can be seen in the detailed description, the present method compensates for this phenomenon and causes the higher-temperature peak to be corrected upwards more than the lower-temperature peak, thus producing accurate data for further analysis.

Special advantages of the present invention are obtained in specific analytical applications. For example. in genotyping applications using high resolution melt protocols for detection of single nucleotide polymorphisms, the sequence difference between analyzed nucleic acids are minimal and the difference in melting temperature are typically less than 2 °C. In this type of application the distance between melting peaks is partially dictated by the natural nucleotide sequence of the target template. This results in partially overlapping peaks in subsequent melting curve spectra. When analyzing mRNA expression in a sample using a competitive PCR technique such as Genome-Controlled RT-PCR it is preferable that the difference in the nucleotide sequence of wild type and reference PCR amplicons is minimal in order to ensure close-to-identical amplification efficiency of the co-amplified templates. Generally, in this type of assay, competitively

amplified templates of less than 100 nucleotides in length, typically differing in nucleotide composition by 3-7 bases, which results in a 2-5 °C difference in melting temperature of the respective PCR amplicons are quantified. In this type of application the distance between the specific melting peaks can be adjusted to some extent. The size of the peaks, however, vary considerably depending on the original amounts of wild type and reference templates in the sample. Significant variations in peak size can cause overlapping of peaks even when the distance between adjacent peaks in a melting curve spectrum is greater than 3 °C. Regardless if the overlapping of adjacent peaks is caused by a small difference in melting temperature or by significant variations in peak size, such peaks can be efficiently and conveniently analyzed using the aspects of the present invention.

The second aspect of the present invention is a method for calculating the area of the separate melting peaks in a spectrum by a curve fitting algorithm to obtain a precise mathematical estimation of the areas of the independent, partially overlapping melting peaks in the spectrum. The peak areas based on curve fits are used instead of actual peak areas to calculate the relative amounts of PCR amplicons present in the sample subsequent to the amplification reaction.

The benefit of sequentially estimating peak areas by curve fitting, rather than from the actual peaks is that the areas of partially overlapping peaks in a spectrum can be precisely estimated. This is especially beneficial in situations where there is a overlapping of adjacent peaks due to considerable difference in areas of adjacent peaks and/or when the difference in melting temperature between separate amplicons is small. This results in accurate quantitative measurement of relative peak areas, which is fundamental for quantitative measurement of gene expression using competitive amplification techniques. Typically, in this type of assay, the melting temperatures of the specific PCR amplicons in the samples are known. This allows for pre-determining melting windows, where upper and lower temperature boundaries for the independent specific melting peaks are defined. This allows for automation of the assay analysis by automated detection and curve fit of multiple specific melting peaks present in the same spectra.

In competitive PCR techniques, the wild type template is co-amplified with one or multiple reference templates in the same reaction, with the same pair of PCR primers. The amplification of these templates is equally affected by variations in amplification efficiency between separate reaction vials. Thus, the amount of wild type template originally present in the sample prior to amplification can be determined from the ratios of wild type and reference PCR amplicons present after amplification. We have observed that a calibration curve consisting of serial samples with a linearly increasing amount of wild type template and a constant amount of reference template generated with this type of assay will have a sigmoid shape. In order to maximize the dynamic range of the assay it is beneficial to perform a mathematical transformation of the quantitative data that renders the calibration curve linear.

The third aspect of the present invention is a method for calculating the absolute amount of nucleic acid template molecules present in a sample prior to amplification. A calibration curve is generated for each of the analyzed genes by performing competitive amplification of samples with linearly increasing amounts of wildtype template and a constant amount of reference template or vice versa. Mathematical correction of the raw melting curves and estimation of the relative peak areas using a curve fitting algorithm can be performed as described above. A Logit-Log transformation of the obtained data is performed and a calibration curve is generated. There is a linear dependency between the *Logit (p)* value (*Log (p/(1-p))*) of the relative peak area estimates and the logarithm *Log R* of the relative amount of wild type and reference templates *R*. The Logit-Log transformation linearizes the calibration curve and thus significantly increases the dynamic range of the assay. After the transformation, the necessary parameters, that is, the linear regression slope and y-intercept values are calculated and stored in a database separately for each gene. When the absolute amount of reference template is known, the unknown amount of wild type template molecules present in a sample prior to PCR amplification, can be calculated from the relative melting peak areas of wildtype and reference amplicons, using the gene-specific slope and y-intercept values.

Logit-Log transformations have been extensively utilized for similar purposes in immunological assays. An example of this is presented in WO publication 2002/076343. We have observed that this type of mathematical transformation is applicable to assays based on PCR, in particular competitive PCR, despite the exponential nature of PCR amplification. In such assays a constant amout of a reference DNA template competes with a wild type template for DNA polymerase binding sites. This situation has proven to be analogous to an immunological assay, in which, a constant amount of labelled antigen competes with the antigen in the sample for antibody binding sites. It should be noted that the present idea of using the Logit-Log transformation in competitive PCR can be extended to other non-immunological PCR-employing applications for calculating amounts of template molecules, in particular in situations where two or more analytes compete for binding sites. This is the case with two templates and a polymerase in competitive PCR.

Each of the three main aspects of the present invention discussed above are independently beneficial for relative quantification of nucleic acids using melting curve analysis. These three aspects of the present invention can be used independently, or together in groups of two or three to gain maximal precision of the quantitative analysis. All three aspects of the present invention can be used in any melting curve assay using fluorescent or luminiscent detection in which the sample comprises two or more nucleic acid molecules with separate specific melting temperatures. Examples of such techniques are quantitative and/or competitive PCR techniques such as those described in publications*: Wang, A.M. et al., Proc. Natl. Acad. Sci. 86, 9717-9721 (1989), Becker-André, M. & Hahlbrock, K. Nucl. Acids Res. 17, 9437-9446 (1989), Gilliland, G. et al., Proc. Natl. Acad. Sci. 87, 2725-2729 (1990)* and *Stenman, J. et al., Clin. Chem. 52:11 1988–1996 (2006)*. Additional examples are various genotyping applications, examples of which are given in WO 2007/035806 and techniques for quantitative analysis of differentially expressed alleles based on single nucleotide polymorphisms as described in *Kuokkanen M. et al., Gut 52, 647-652 (2003)* and techniques for quantitative analysis of differentially expressed highly homologous genes as described in *Stenman, J et al., Nat.*

Biotechnol. 17, 720-722 (1999). The first and second aspects, or portions thereof, can also be employed for samples containing only a single nucleic acid.

The invention can be used, for example, for the determination of relative amounts of mRNA transcripts in a cell or tissue sample. When using the Genome-Controlled RT-PCR technique for this purpose, sequence-modified cDNA templates are co-amplified competitively with one or several reference DNA templates using the same PCR primers. In this technique it is possible to use Genomic DNA (gDNA), synthesized or cloned DNA oligomers or polymers as a reference DNA template in order normalize for the differences in amplification reaction efficiency between separate reaction vials.

By using melting curve analysis to measure the relative amount of sequence-modified cDNA and reference DNA amplicons during PCR cycling or after amplification, the relative amounts of mRNA transcripts originally present in the sample can be determined. As cDNA and reference DNA templates are amplified using the same primers in the same reaction vials, the relative amount of the respective amplicons remain unchanged even when amplification reactions are run to the plateau phase. This can be utilized by running reactions to the plateau phase in order to bring the signal into the measuring range of the assay regardless of the amount of starting template present in the sample. This type of technique is presented extensively in WO publication 2005/116248, the essential contents of which are incorporated herein by reference.

Analogously, the invention can be used in an assay for determination of relative amounts of highly homologous mRNA or DNA templates naturally present in a sample by reverse transcription of mRNA templates and competitive co-amplification of cDNA and / or DNA templates and quantitative detection by melting curve analysis.

Next, embodiments and advantages of the invention are described with the aid of the attached drawings.

BRIEF DESCRIPTION OF DRAWINGS

Figs. 1a – 1d show the determination of a correction factor for the melting curve correction function according to one embodiment of the invention.

Fig. 2 shows exemplary melting peaks generated by calculating the negative derivative of the measured melting curve $-dF_{meas}/dT$ corrected melting curve $-dF_{corr}/dT$.

Figs. 3a – 3f illustrate the fitting of gauss functions to corrected melting peaks of two amplicons present in the sample.

Figs. 4a and 4b illustrate, respectively, a Log-Log plot of a standard curve and Logit-Log plot which linearizes the entire range of the standard curve (gene: dd3).

DETAILED DESCRIPTION OF EMBODIMENTS

As discussed above, the method according to first aspect of the invention comprises analyzing a nucleic acid melting curve measured from a sample, the melting curve comprising a sum signal of at least two nucleic acid melt signals and a background signal as a function of temperature. The method further comprises optimizing at least one constant in a temperature-dependent exponential correction function so as to minimize the variation of the nucleic acid melting curve at a temperature region where the target nucleic acids in the sample remain essentially double stranded, and generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal by applying said exponential correction function over the region of the measured melting curve where the strands of the nucleic acids dissociate. According to further aspects, the invention relates to a curve fitting algorithm for precise estimation of melting peak areas and a mathematical transformation for linearization of calibration curve data to enhance the linear measuring range of a competitive PCR assay. In summary. the invention provides powerful tools for analyzing PCR-amplified sample containing two or more different nucleic acids having similar but distinguishable melting temperatures.

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Definitions

The term ”PCR amplicon” means the PCR product of any PCR-amplifiable template molecule. In the context of competitive PCR, the term generally means the PCR product generated by amplification of a specific cDNA or DNA template. In techniques based on competitive PCR, separate amplicons are derived from wild type and reference templates that are co-amplified in the same reaction with the same primers.

The term ”wild type template” means a nucleic acid template that is being analyzed. Wild type cDNA templates are generated during reverse transcription of mRNA species present in the original sample, whereas, a wild type DNA templates occur naturally in the original sample. The amount of wild type template is usually unknown prior to analysis.

The term ”reference template” means a nucleic acid template that is included in each amplification reaction and serves to normalize for tube-to-tube variations in amplification efficiency. In the context of Genome-controlled RT-PCR, the reference DNA templates comprise genomic DNA contained in or added to the sample, or a pool of gene-specific templates such as cloned or synthesized DNA oligo- or polynucleotides for each of the analyzed genes. The wild type and reference templates in an individual amplification reaction are co-amplified in a competitive manner, using the same primers. Gene-specific synthetic or cloned reference templates can be concatenated by ligation or other means to form a single polynucleotide that serves as a universal reference template for several genes.

The term ”co-amplification” means simultaneous amplification of two or more templates. For example in competitive PCR, the co-amplified templates may be cDNA and/or DNA templates with differing nucleotide sequences within the generated amplicons. A pre-requisite for co-amplification as the term is used in this patent application is that templates have identical nucleotide sequences at the primer-binding sites used in the amplification reaction and that amplification of said templates is carried out in a competitive manner with the same primers and other reagents, in the same reaction vial.

Correction of measured melting curve

As briefly explained above, in the first aspect of the invention, the background fluorescence is estimated from the initial portion of the raw melting curve prior to the specific nucleic acid melting transition where the amplified nucleic acids remain essentially double-stranded. An exponential correction factor is calculated on the basis of the initial portion of the melting curve. The entire measured fluorescence curve is thereafter multiplied by a function of temperature in which said exponential correction factor is incorporated. This transformation renders the average derivative of the background signal close to zero. If the melting experiment includes several samples, as is typically the case, the transformation is performed separately for each independent sample taking into account the amount of signal present in each individual sample.

Thus, in general terms, the method according to one embodiment comprises the following steps:

- A correction factor K, which is the base in an exponential correction function, is optimized in order to render the change in background signal intensity close to zero over the range prior to the specific nucleic acid melting transition.
- The correction function is applied to every temperature point in the melting curve to generate a corrected melting curve.

Optimization of the correction factor K is performed using the data of the measured melting curve at a temperature range prior to the specific nucleic acid melting transition. The minimum range width required depends on the frequency of the temperature data points obtained, which is a functional feature of the specific instrument used. Basically, a slope can be formed using a minimum of two data points, but preferably three to five data points are used to obtain more reliable results. The range typically has a width of at least 1 °C, in particular at least 3 °C, and preferably at least 6 °C, In this fashion, the fluorescent signal used for estimation of background fluorescence is not affected by

melting of the specific nucleic acids in the sample. This ensures that the proportion of background fluorescence in the sample is correctly estimated.

The present method, renders the background fluorescence close to zero and enhances the dynamic range at the specific nucleic acid melting transition. We have observed that this type of correction gives a precise estimation of the sample-derived signal obtained from temperature-specific dissociation of the DNA strands of the PCR amplicons in the sample. When the correction is performed for two adjacent melting peaks with seemingly identical areas, the correction renders the area of a peak obtained at a higher melting temperature larger than that of a peak obtained at a lower melting temperature. This is due to the fact that the fluorescent signal per molecule of DNA is temperature-dependent.

According to a preferred embodiment, the correction obeys the formula

$$F_{corr}(T) = \frac{1+K^{dT}}{2} F_{meas}(T) \quad (1)$$

which can also be written in the following form:

$$F_{corr}(T) = \left(\frac{1+Ae^{BT}}{2}\right) F_{meas}(T) \quad (2)$$

where $F_{meas}(T)$ is the measured melting curve, $dT=T-T_0$ and constants A and B are defined as follows: $B = ln\ K$ and $A = e^{-BT_0}$.

Even more generally, the correction can be formulated as

$$F_{corr}(T) = (C_1 + C_2 e^{C_3 T}) F_{meas}(T) \quad (3)$$

where C_1, C_2 and C_3 are constants. The correction function can be calculated according to the above-described optimization principle for C_3 after fixing C_1 and C_2.

As appreciated by persons skilled in the art, the exponential correction term can be mathematically formulated in various ways.

The parameter T_0 can be relatively freely chosen from the range before the melting transition, as it only defines the starting point of correction and the correction equation is designed to yield $F_{corr}(T_0) = F_{meas}(T_0)$.

A typical melting curve spectrum is generated by calculating the negative derivative of the corrected raw melting curve $-dF/dT$. A benefit of the invention in comparison with earlier techniques is that the entire melting curve is corrected for the effect of background fluorescence which allows for precise measurement of multiple specific melting peaks over the entire range of the melting curve.

In practice, the fluorescence measurements are carried out at discrete steps and the measurement results stored in an array. The calculation of the correction factor can thus be performed by discrete mathematical methods by a computer program product designed for this purpose or in a spreadsheet program.

In the following, calculation of the corrected melting curve using the abovementioned formula is described in detail.

Figs. 1a – 1d illustrate the optimization of the correction factor K using the formula (1) above. The factor is gradually increased to find the optimal value, which normalizes the corrected curve at the initial part of the curve. Figs. 1a shows a situation where no correction is performed ($K = 1$). Fig. 1d shows an optimal correction ($K = 1.077$) which normalizes the non-dissociating region of the melting curve. This value is chosen for the K-value for correction of the whole curve using the same formula. Minimizing the sum of deviations of the curve from normal level can be implemented using first-order sums or e.g. least squares method.

It is also understood that other method for finding the optimal K-value can be used. These may include e.g. iterative or other computational methods.

Fig. 2 shows the first negative derivatives of an uncorrected and corrected melting curve. As can be seen from the data, the correction causes the relative heights of the peaks of different amplicons to change. In the uncorrected data, the ratio of peak heights is 184.5/365.5=0.50 and in the corrected data the ratio is 438.7/691.6=0.63. As the rise in temperature causes the measured signals to weaken, the correction can be found to rectify the data in this respect. Thus, the following calculation of the proportions of the amplicons in the sample is more accurate.

The analysis is preferably performed utilizing a computer program that is run in the central processing unit of a computer. The following preparational steps can be performed prior to the analysis:

- The temperature and fluorescence data are retrieved from the memory unit of the computer or directly from the instrument performing the sample melting and signal aquisition,
- the melting temperature range of the wild type and the reference amplicons and preferably the identification number or name of the analysis are retrieved from a database or recieved from the user, and
- appropriate start and end points are determined from the temperature and fluorescence data if these include excessive data points.

Calculating the proportions of amplicons

The relative amounts of amplicons in the sample can be calculated from the corrected melting curve using curve fittings representing the amounts of amplicons, rather than directly using the measured first derivative melting curve peaks.

In this method, a curve fit is performed for the first peak in the corrected first derivative melting curve spectrum in order to obtain a mathematical estimation of the area of the first melting peak. Subsequently the difference of the corrected first derivative melting

curve and said curve fit is calculated to obtain a residual corrected melting curve. A second curve fit is performed for the second peak in the residual melting curve to obtain a mathematical estimation of the area of the second melting peak. This procedure is repeated as many times as required in order to estimate the areas of each of the specific melting peaks in the spectrum. The areas of the curve fits for the separate melting peaks are used to determine the relative concentrations of PCR amplicons present in the sample. In competitive PCR such as the Genome-Controlled RT-PCR technique the relative concentrations of wild type and reference DNA amplicons reflect the amount of wild type cDNA and reference DNA originally present in the sample prior to amplification. In a modification of this technique two or multiple separate reference DNA templates added at different known concentrations to the sample can be co-amplified with the wild type cDNA template. In this manner a sample-specific calibration curve can be obtained from each individual amplification reaction, which further increases the quantitative precision of the analysis.

In the Genome-Controlled RT-PCR technique PCR amplicons are typically in the range 30-70 nucleotides in length. The melting temperature of reference DNA templates is typically modified by a nucleotide substitution without altering the length of the subsequent PCR amplicons. This results in a difference in the melting temperatures of wild type and reference DNA PCR amplicons of less than 5° C. When using competitive PCR and melting curve analysis for genotyping applications or for quantification of the expression level of differentially expressed alleles based on single nucleotide polymorphisms, the difference in melting temperature between the amplicons are typically less than 3° C. In addition, there is a degree of inherent temperature variation across the thermal block of many instruments currently being used for nucleic acid melting. As a result the melting peaks of a specific nucleic acid will appear at slightly different temperatures in the melting curve spectrum, depending on where the sample has been situated in the block.

Adjacent melting peaks that overlap cause significant skewing. In particular, a peak adjacent to a significantly larger peak often appears only as a shoulder on the larger peak.

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In such a situation the estimation of actual peak areas is difficult and imprecise. The curve-fitting procedure of the present invention allows precise estimation of the relative amount of amplicons present in the sample, in particular when there is partial overlapping of adjacent peaks and/or when difference in peak areas is large.

The method of the present invention can be processed in a separate computer, which typically forms part of a quantitative PCR system, or in the central processing unit of the PCR cycling or melting curve instrument itself.

Example

This example illustrates the various aspects of the invention by describing the main steps of a melting curve analysis wherein the sample contains two nucleic acids having different melting temperatures.

a) Correction of the melting curve

Below is presented an example of calculation of the correction from two separate temperature points on the melting curve using the principles disclosed above. Values of a pre-specified temperature range where no dissociation of specific nucleic acids occur are used for generating an optimal correction of the entire melting curve. Herein, $T_0=59,3$ *(°C)*, corresponding to the starting temperature for recording of data in the measurement.

Table 1. Example of correction of a measured melting curve (at non-dissociating region)

point	Temp	F_{meas}	dT	(1+K^dT)/2	F_{corr}
1	59,3	**0,1536**			**0,1536**
12	62,2	0,1372			
13	62,5	**0,1355**	3,2	1,1328	**0,1535**
24	65,8	0,1175			
25	66,1	**0,1155**	6,8	1,3249	**0,1530**

Table 1 demonstrates that using the optimized correction factor K in the correction function (1+K^dT)/2 renders the corrected fluorescence values close-to-equal at temperatures below the melting transition of the target nucleic acids in the samples. After correction the raw (non-derived) melting curve will be horizontal, i.e. normalized at the non-dissociating region. The behaviour of the corrected curve at the dissociating region and after that is shown in the drawings.

b) Determination of melting peak areas

Measurement of the areas of the specific melting peaks can be carried out by

- determining the location of maximum rate of change in corrected signal strength, e.g. by finding the maximum value of the derivative for the first nucleic acid occurring in the melting curve (for this purpose pre-determined melting temperature ranges for the respective amplicons can be utilized),
- performing a curve fit for the first melting peak, for example by optimizing the width of a chosen curve form (i.e. gaussian) to fit the peak, i.e., until a minimum for the difference of the curve and the first melting peak (i.e., residual curve) is found (this is illustrated in Figs. 3a and 3b (showing a non-optimal fit) and Fig. 3c (showing an optimal fit)),
- when the width of the curve has been determined to fit the first nucleic acid melting peak, the difference of *the measured value – value of said curve* in each data point of the entire derivative melting curve is calculated to generate a residual melting curve (Fig. 3c),
- determining from the residual melting curve the the peak of the second nucleic acid by finding the maximum change of rate of the signal, e.g. by finding or the maximum value of the derivative for the second nucleic acid occurring in the melting curve (for this purpose pre-determined melting temperature ranges for the respective PCR amplicons can be utilized),
- performing a curve fit by optimizing the width of a curve (i.e. gaussian) to fit the peak of the second nucleic acid in the first derivative melting curve by using said

maximum value as the maximum for the curve (Figs. 3e (non-optimal fit) and 3f (optimal fit)),

- if more than two nucleic acids are present, repeating these steps are until a curve fit has been performed for each of the specific nucleic acid melting peaks in the sample,
- optionally, first derivative melting curves and/or the goodness of the curve fit and/or the calculated residual melting curves are graphically displayed and,
- calculating the areas of each of the fitted curves and using these areas to estimate the relative amounts of the respective amplicons present in the sample.

Automation of the curve fittings is generally possible using the above algorithm. However, the fittings can also be performed fully or partly manually, if desired.

c) Calculating the absolute amounts of nucleic acids in the sample

In one embodiment of the present invention a calibration curve is generated in order to enable quantification of the unknown absolute amount of wildtype template molecules present in the sample prior to amplification. This calibration curve is generated by performing amplification of samples with known wild type / reference template ratios at constant increments. A Logit-Log transformation where the logit of a number p between 0 and 1 is given by the formula $\text{logit}(p) = \log(p) - \log(1-p)$ is performed to linearize the sigmoidal calibration curve. The linear regression slope and y-intercept values of the linearized calibration curve are calculated and stored in a data base. These values are used to calculate the unknown absolute amount of wild type template present in the sample prior to amplification from the Logit-Log transformed ratio of wild type and reference amplicon-derived melting curve areas in a sample. This is achieved by:

- co-amplifying a linearly increasing amount of wild type template with a constant amount of reference template in serial samples. For this purpose synthetic or

cloned DNA oligo- or polynucleotides with the same nucleotide composition as the wildtype and reference templates can be used,

- performing melting curve analysis of the amplicons to obtain the ratio of cDNA and reference DNA-derived melting curve areas from each of the serial samples to generate a calibration curve (preferably, but not necessarily, the melting curve correction and/or melting peak area calculation according to other aspects of the invention are used),
- performing Logit-Log transformation of the relative curve areas in order to linearize the calibration curve, (Fig. 4a shows the sigmoidal calibration curve (gene: dd3) as a Log-Log graph, fig 4b shows the calibration curve after Logit-Log transformation)
- calculating the linear regression slope and y-intercept values from the calibration curve and storing these in a database, separately for each gene included in a subsequent analysis,
- using the gene-specific slope and y-intercept values to calculate the unknown absolute amount of wild type template molecules originally present in a sample, from the the sample-specific ratio of wild type and reference amplicon-derived melting curve areas

As can be seen from Fig. 4a, the Log-Log plot of the standard curve is typically sigmoidal, but the Logit-Log function, shown in Fig. 4b linearizes the entire range of the standard curve (gene: dd3). Thus, the linear dynamic range of the analysis is increased.

The basic mathematical principles of Logit-Log transformation, however, in the context of radioimmunological assay, is presented in *Rodbard D. et al, Mathematical analysis of kinetics of radioligand assays: improved sensitivity obtained by delayed addition of labeled ligand. J Clin Endocrinol Metab. 1971 Aug;33(2):343-55.*

The embodiments described above and shown in the attached drawings are given for illustrative purposes and are not intended to limit the scope of the present invention which is defined in the following claims.

Claims:

1. A method for analyzing a sample containing at least one nucleic acid using a nucleic acid melting curve measured from the sample, the melting curve comprising a sum signal of nucleic acid melt signal and a background signal as a function of temperature, the method comprising

- optimizing at least one constant in a temperature-dependent exponential correction function so as to minimize the variation of the nucleic acid melting curve at temperature region where the nucleic acids in the sample remain essentially double stranded, and
- generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal by applying said exponential correction function over the region of the measured melting curve, or derivative thereof, where the strands of the nucleic acids dissociate.

2. The method according to claim 1, wherein the measured melting curve is multiplied with the exponential correction function in order to obtain said corrected melting curve.

3. The method according to claim 1 or 2, wherein the corrected nucleic acid melting curve is generated by multiplying the measured nucleic acid melting curve at the region of the measured melting curve where the strands of the nucleic acids dissociate by an exponential function including a term K^T, or a mathematical equivalent thereof, wherein K is a constant correction factor being optimized using data from the region of the melting curve where no dissociation of target nucleic acids occurs.

4. The method according to any of the preceding claims, wherein the correction obeys the formula

$$F_{corr}(T) = \frac{1+K^{dT}}{2} F_{meas}(T),$$

where $F_{corr}(T)$ is the corrected melting curve, $F_{meas}(T)$ is the measured melting curve, $dT=T-T_0$, where T_0 is a predefined starting temperature and K is a constant optimized

using the measured melting curve at the temperature region where the target nucleic acids in the sample remain essentially double stranded.

5. The method according to any of the preceding claims, wherein the optimization of the constant in the correction function is performed over a temperature range having a width of at least 1 °C, in particularly at least 3 °C, preferably at least 6 °C.

6. The method according to any of the preceding claims, wherein a derivative of the corrected melting curve is calculated in order to obtain a melting peak curve.

7. The method according to any of the preceding claims, wherein the nucleic acid melting curve is measured from a sample processed by PCR amplification, in particular competitive PCR amplification.

8. The method according to any of the preceding claims, wherein the sample contains two or more different nucleic acids having different melting temperatures.

9. The method according to claim 8, wherein the nucleic acid melting curve is measured from a Genome-Controlled RT-PCR sample containing a wild type cDNA and reference DNA nucleic acids.

10. The method according to claim 8 or 9, wherein the difference of the melting temperatures of the at least two different nucleic acids is less than 10 °C, in particular less than 5 °C, preferably 0.5 – 5 °C.

11. The method according to any of claims 8 - 10, wherein the length of each of the at least two nucleic acids is less than 500 nucleotides, advantageously less than 100 nucleotides, in particular 30 – 70 nucleotides.

12. The method according to any of claims 8 – 11, further comprising the steps of

- calculating a first derivative of the corrected melting curve in order to form a melting peak curve,
- determining the areas of at least two melting peaks of the melting peak curve,
- calculating the proportions of nucleic acids in the sample using said areas.

13. A system for analyzing melting properties of nucleic acids, comprising

- means for measuring a nucleic acid melting curve as a function of temperature, the melting curve comprising a sum signal of nucleic acid melt signal and a background signal,
- computing unit for analyzing the measured melting curve, and
- means for transferring the measured melting curve to said computing unit,

wherein the computing unit comprises

- means for optimizing at least one constant in a temperature-dependent exponential correction function so as to minimize the variation in the sum signal intensity at a temperature region where the nucleic acids in the sample remain essentially double stranded, and
- means for generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal by applying said exponential correction function over the region of the measured melting curve, or derivative thereof, where the strands or the nucleic acids dissociate.

14. The system according to claim 13, wherein the computing unit is adapted to carry out the method according to any of claims 1 – 12.

15. A computer program product stored on a computer-readable medium for analyzing nucleic acid melting curves, the product being configured to

- read measured melting curve data including temperature values and corresponding signal values from a data file,
- optimize, based on said data, at least one constant in a temperature-dependent exponential correction function so as to minimize the variation in the sum signal

intensity at a temperature region where the nucleic acids in the sample remain essentially double stranded

- calculate a corrected nucleic acid melting curve using said exponential correction function over the region of the measured melting curve, or derivative thereof, where the strands or the nucleic acids dissociate.

16. The computer program product according to claim 15, configured to perform the method according to any of claims 1 – 12.

17. A method for determining the proportions of nucleic acids from a melting curve measured from a sample containing at least two nucleic acids, the method comprising

- correcting the measured melting curve by removing the effect of background signal so as to form a corrected melting curve reflecting melting of the target nucleic acids in the sample,
- calculating a first derivative of the corrected melting curve in order to form a melting peak curve,
- fitting mathematical functions, for example gaussian functions, to the melting peaks contained in the melting peak curve,
- estimating the areas of the melting peaks using said fitted mathematical functions, and
- determining the proportions of nucleic acids in the sample using said areas.

18. The method according to claim 17, wherein said fitting is performed by

- fitting a first mathematical function to a first peak found on the melting peak curve for estimating the first melting peak,
- calculating a residual curve by subtracting said first mathematical function from the melting peak curve,
- fitting a second mathematical function or the like to the residual curve for estimating the second melting peak, and

- optionally, repeating the previous steps for estimating further melting peaks that may be present.

19. The method according to claim 18, wherein the step of correcting the measured melting curve is carried out according to any of claims 1 - 12.

20. A method for determining the proportions of nucleic acids from a melting curve measured from a sample containing at least two nucleic acids, the method comprising

- correcting the measured melting curve by removing the effect of background signal so as to form a corrected melting curve reflecting real melting of the sample,
- calculating a first derivative of the corrected melting curve in order to form a melting peak curve,
- estimating the areas of the melting peaks, and
- determining the proportions of nucleic acids in the sample by Logit-Log transformation using said areas.

21. The method according to claim 20, comprising

- providing a calibration curve for quantification of the amounts of nucleic acids, the calibration curve being obtained by from samples with known nucleic acid ratios at at least two known concentrations,
- linearizing the calibration curve using Logit-Log transformation,
- calculating necessary parameters to describe the linearized calibration curve, and
- using said parameters and said estimated areas of the melting peaks to calculate the absolute amount of at least one of the nucleic acids in the sample prior to amplification.

22. The method according to claim 20 or 21, wherein the step of correcting the measured melting curve is carried out according to any of claims 1 - 12.

23. The method according to any of claims 20 – 22, wherein the proportions of nucleic acids are estimated according to any of claims 16 – 19.

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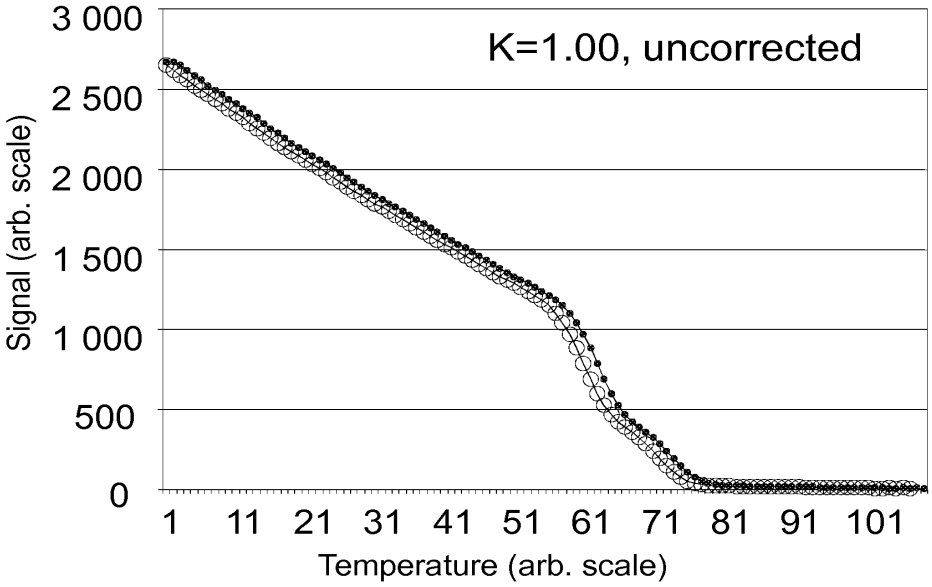

K=1.00, uncorrected
Signal (arb. scale)
3 000
2 500
2 000
1 500
1 000
500
0
1 11 21 31 41 51 61 71 81 91 101
Temperature (arb. scale)

Fig. 1a

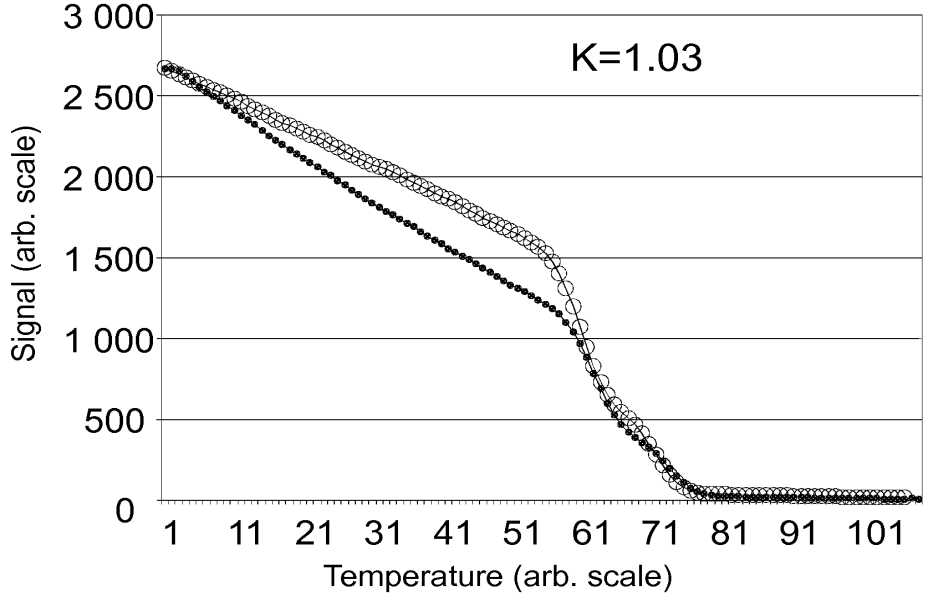

K=1.03
Signal (arb. scale)
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2 500
2 000
1 500
1 000
500
0
1 11 21 31 41 51 61 71 81 91 101
Temperature (arb. scale)

Fig. 1b

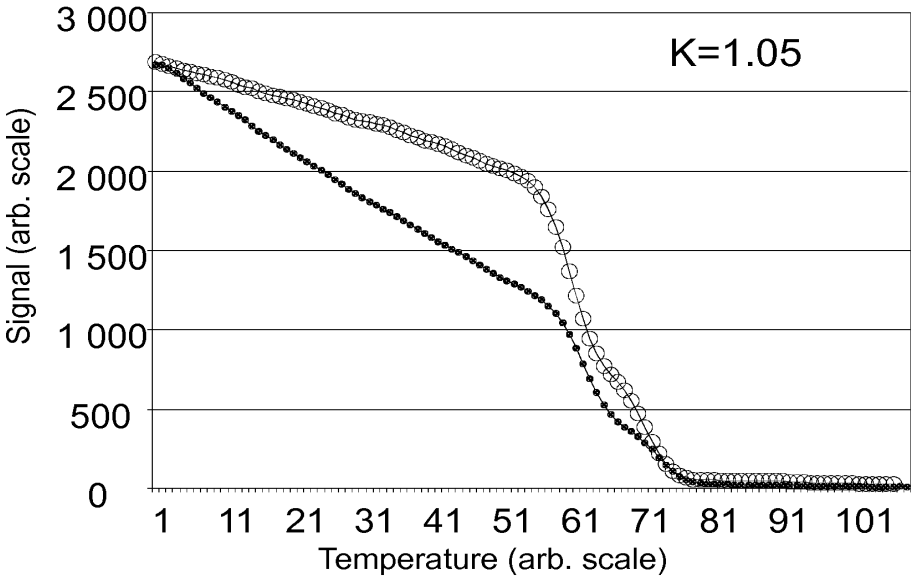

K=1.05
Signal (arb. scale)
3 000
2 500
2 000
1 500
1 000
500
0
1 11 21 31 41 51 61 71 81 91 101
Temperature (arb. scale)

Fig. 1c

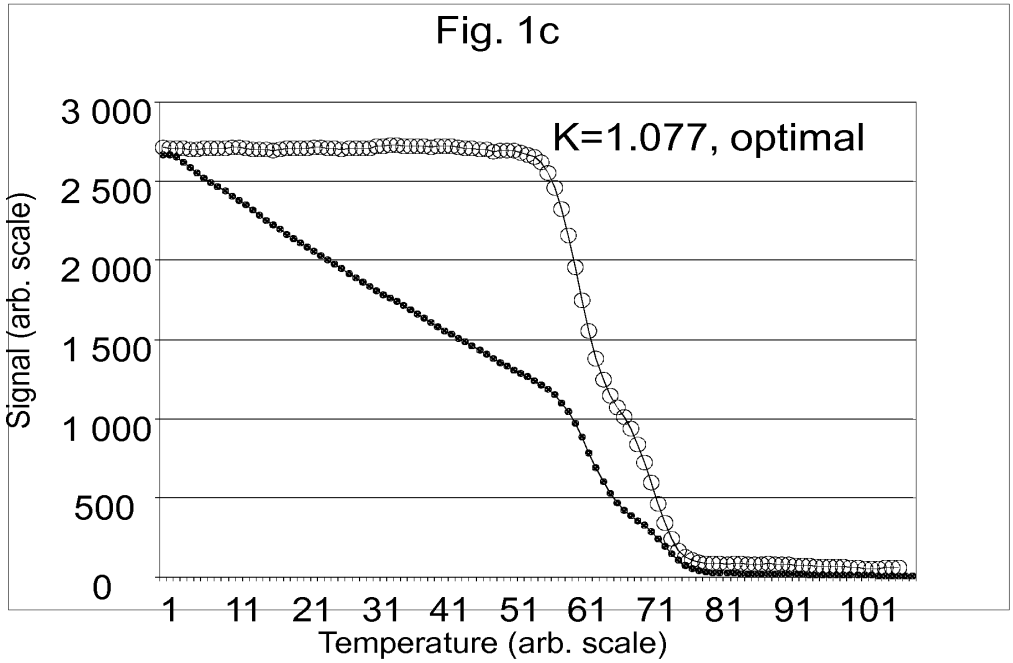

K=1.077, optimal
Signal (arb. scale)
3 000
2 500
2 000
1 500
1 000
500
0
1 11 21 31 41 51 61 71 81 91 101
Temperature (arb. scale)

Fig. 1d

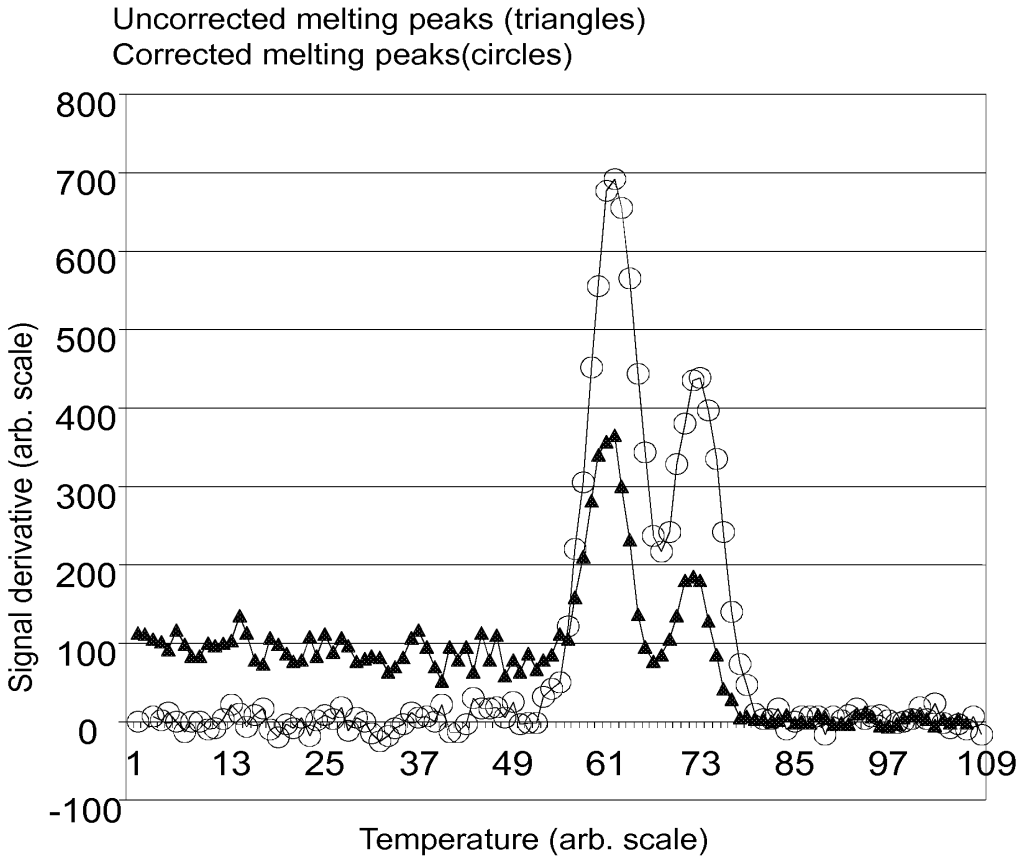

Uncorrected melting peaks (triangles)
Corrected melting peaks(circles)
800
700
600
500
400
300
200
100
0
-100
Signal derivative (arb. scale)
1
13
25
37
49
61
73
85
97
109
Temperature (arb. scale)

Fig. 2

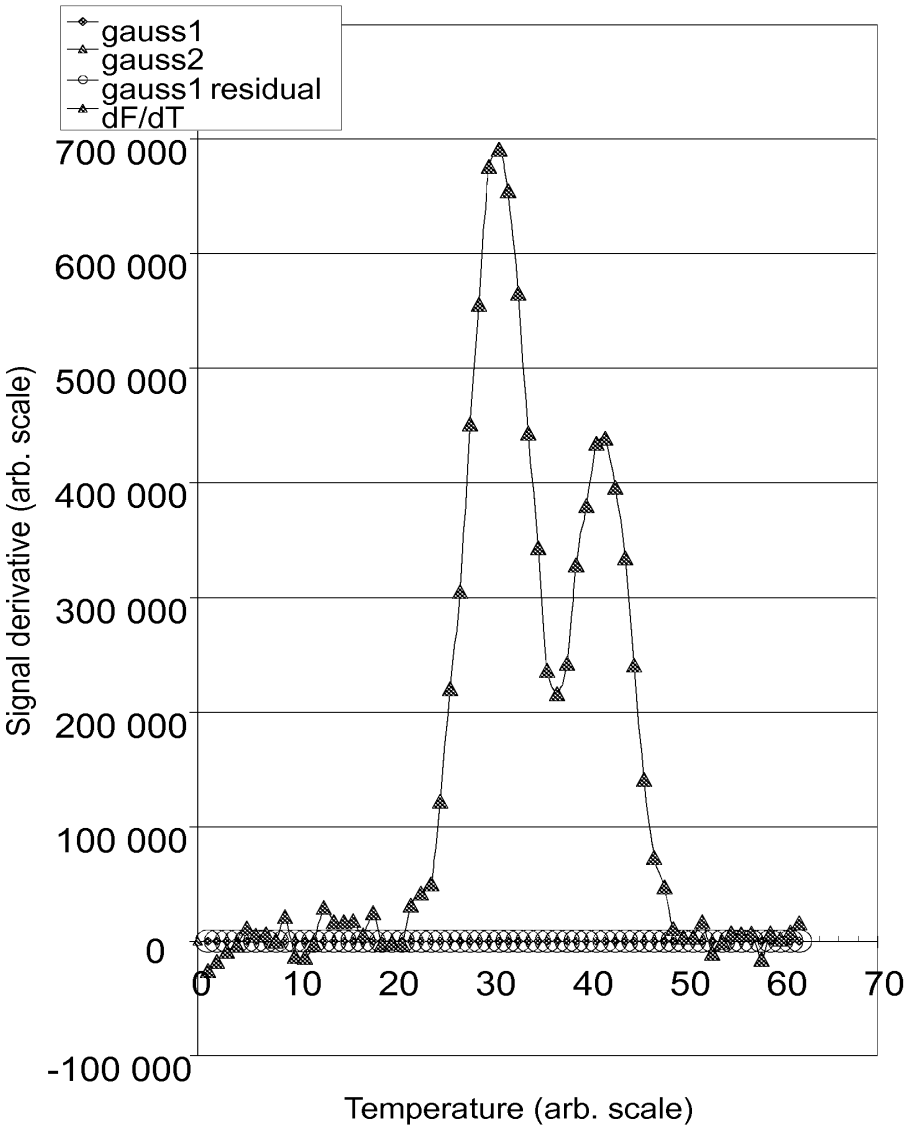

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
10
20
30
40
50
60
70
Temperature (arb. scale)

Fig. 3a

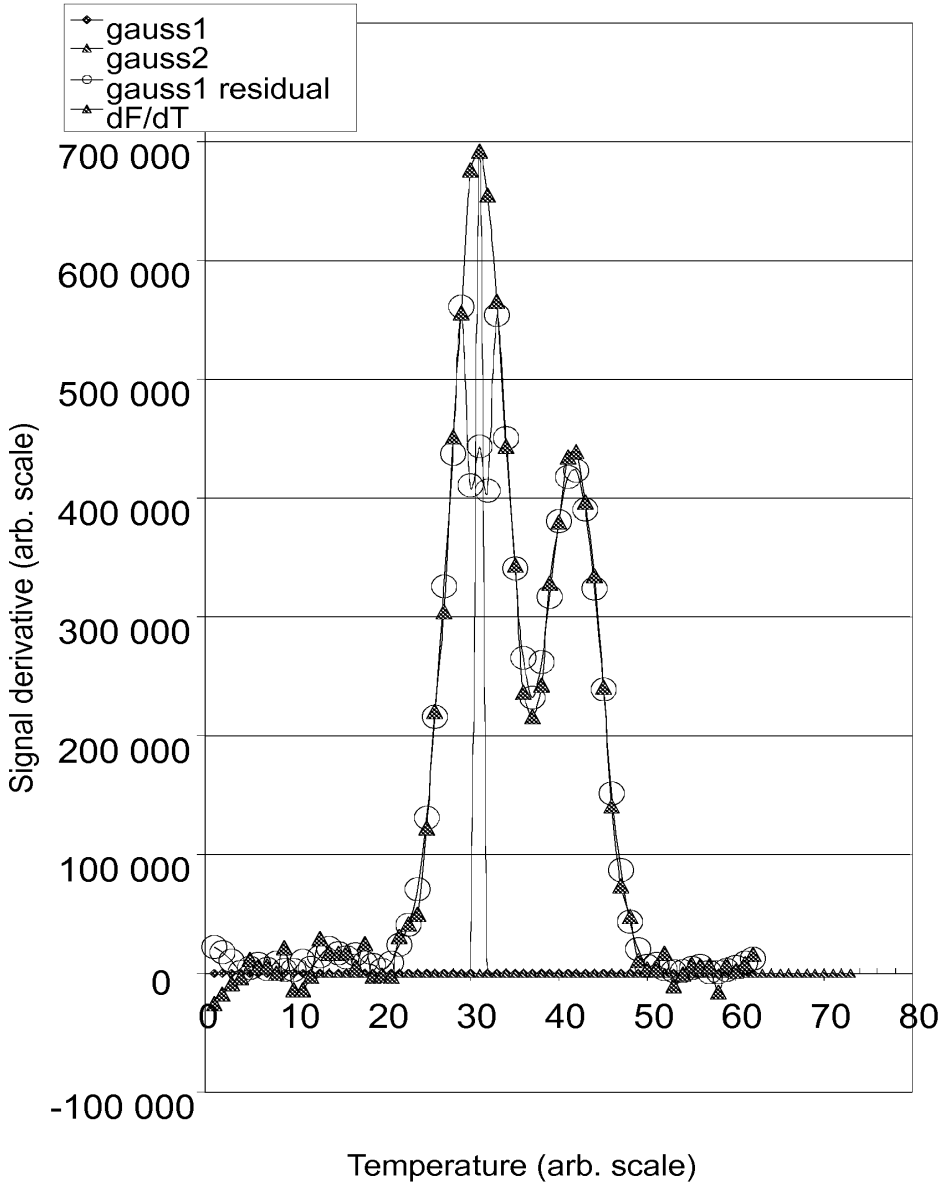

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
10
20
30
40
50
60
70
80
Temperature (arb. scale)

Fig. 3b

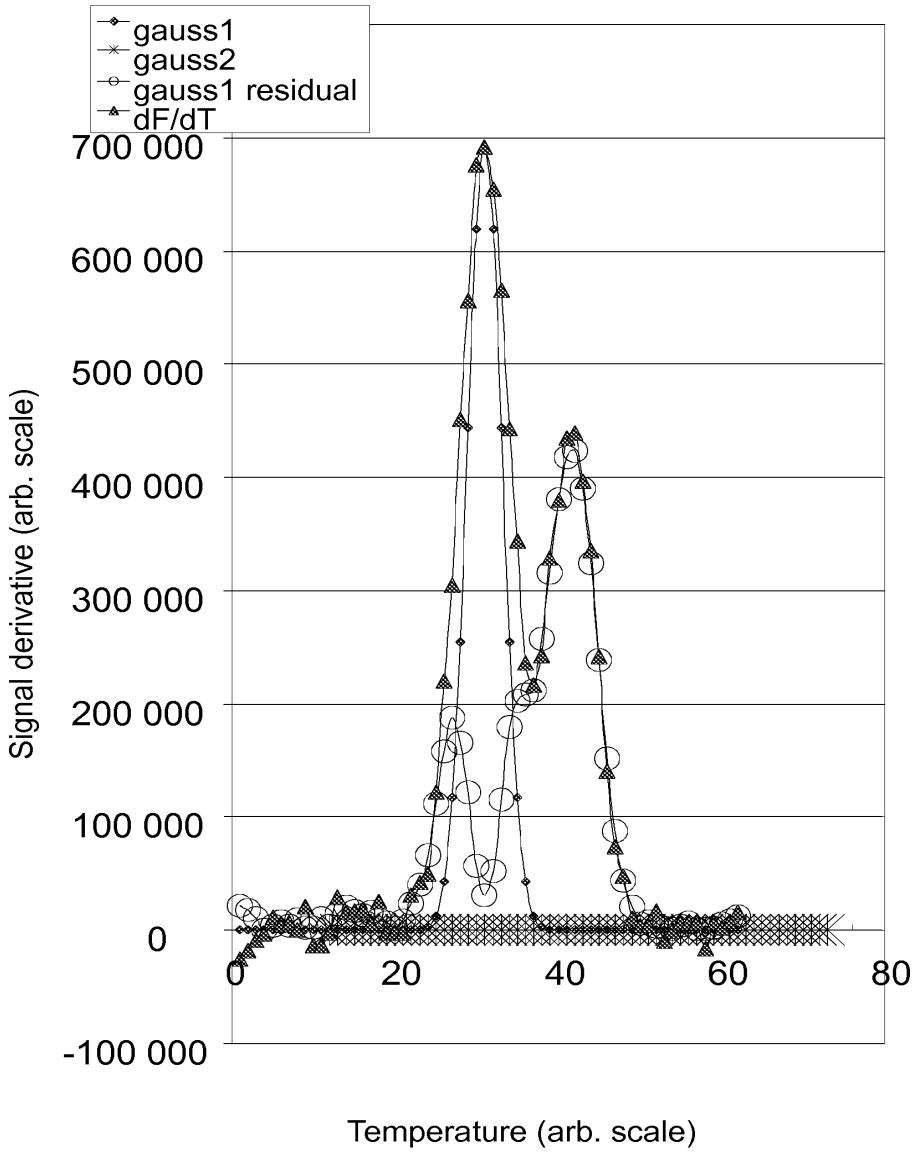

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
20
40
60
80
Temperature (arb. scale)

Fig. 3c

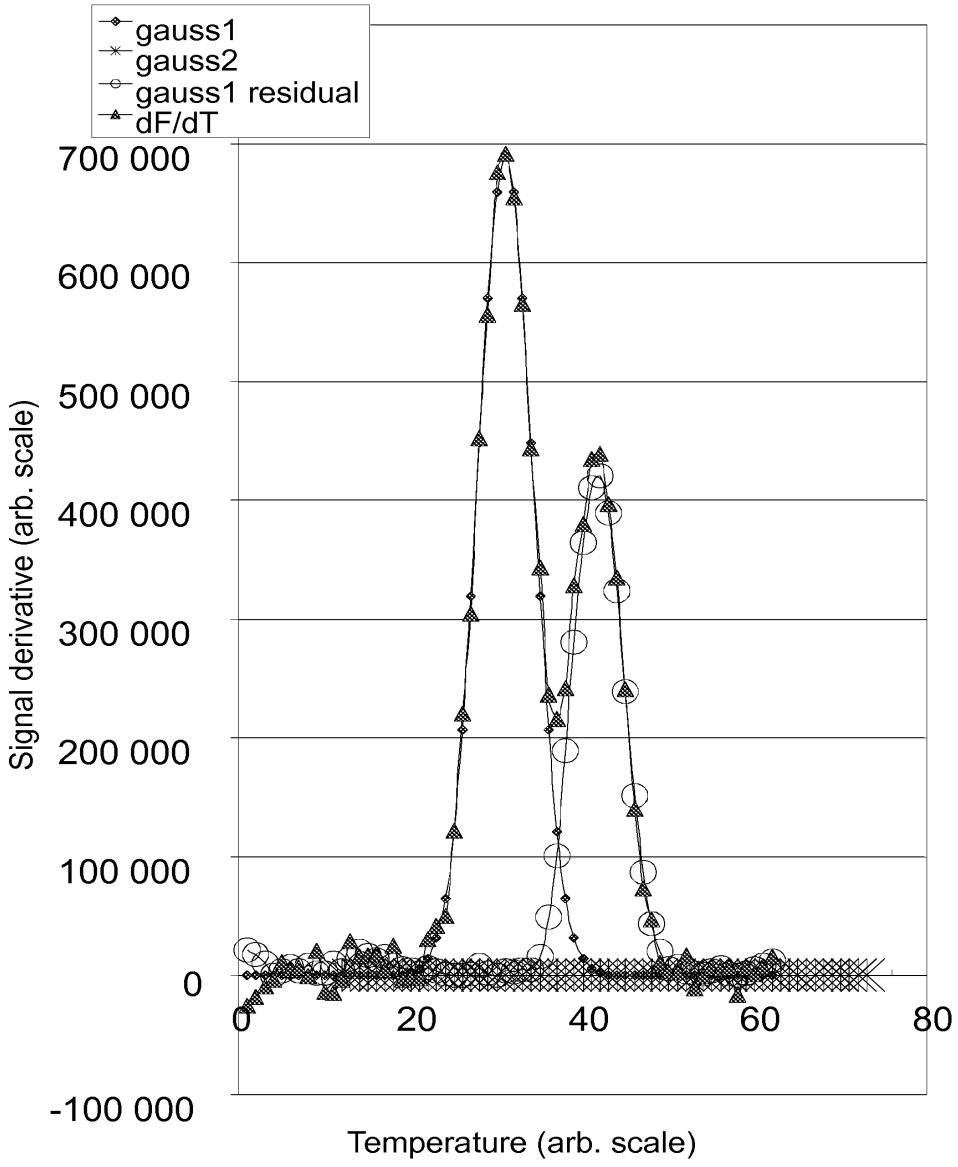

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
20
40
60
80
Temperature (arb. scale)

Fig. 3d

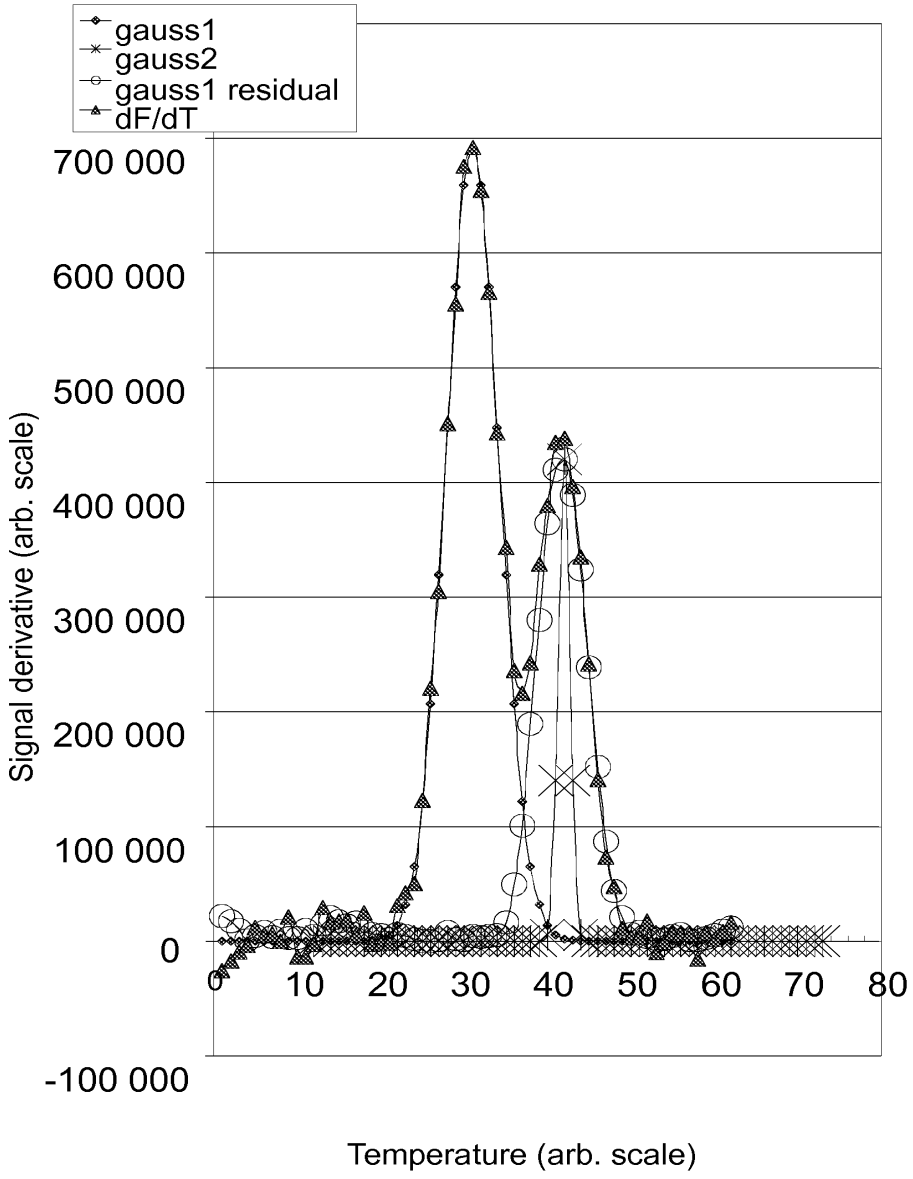

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
10
20
30
40
50
60
70
80
Temperature (arb. scale)

Fig. 3e

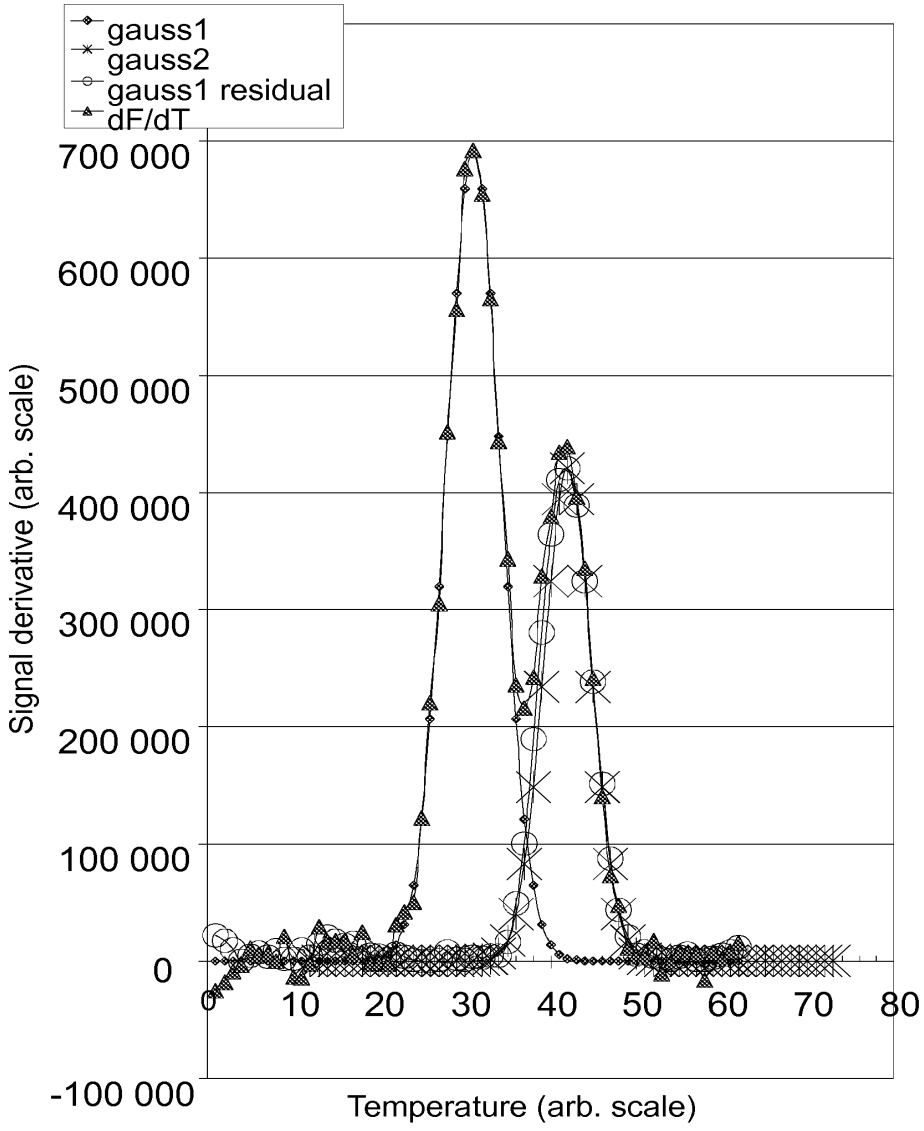

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
10
20
30
40
50
60
70
80
Temperature (arb. scale)

Fig. 3f

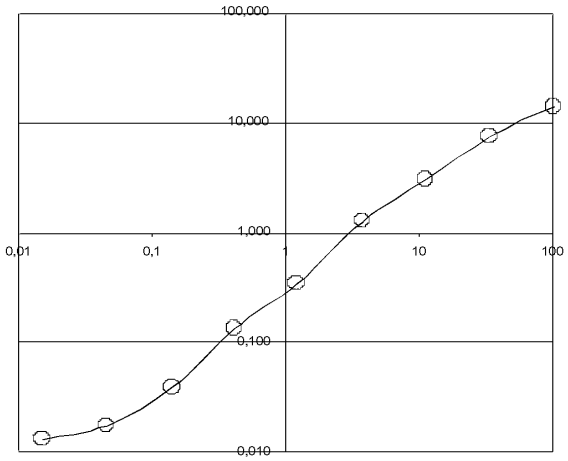


Fig. 4a

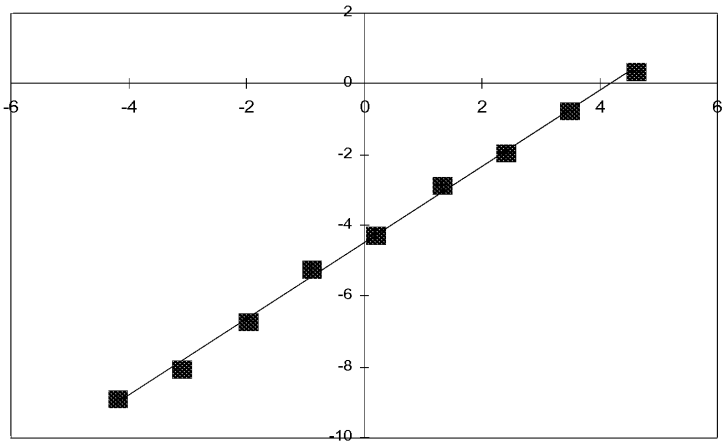


Fig. 4b

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2010/050360

A. CLASSIFICATION OF SUBJECT MATTER
INV. G06F19/00
ADD. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G06F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007/035806 A2 (UNIV UTAH RES FOUND [US]; PALAIS ROBERT ANDREW [US]; WITTWER CARL THOM) 29 March 2007 (2007-03-29) cited in the application * abstract page 5, paragraph 1 - page 7, paragraph 2 page 9, paragraph 2 - page 12, last paragraph figures 1A,1B claim 1 ----- -/--	1-23

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 2010	04/08/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hilbig, Matthias

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2010/050360

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WILHELM ET AL: "SoFAR: Software for Fully Automatic Evaluation of Real-Time PCR Data" BIOTECHNIQUES, INFORMA LIFE SCIENCES PUBLISHING, WESTBOROUGH, MA, US, vol. 34, no. 2, 1 January 2003 (2003-01-01), pages 324-332, XP003024150 ISSN: 0736-6205 the whole document	1-23

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/FI2010/050360

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Form PCT/ISA/210 (patent family annex) (April 2005)

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 1 of 2

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	NEGIN, RUSSELL SCOTT
Attorney Docket Number	38075-716.301

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
/RSN/	001	US-20020146734	10-10-2002	ORTYN; William E. et al.	
/RSN/	002	US-20080124705	05-29-2008	KRAMER; Fred R.	
/RSN/	003	US-20100151443	06-17-2010	XIANG; Qing et al.	
/RSN/	004	US-20110151550	06-23-2011	SAGNER; Gregor et al.	

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/RSN/	001	WO-03002979-A2	01-09-2003	ILLUMINA INC [US], et al.		☐
/RSN/	002	WO-2013096851-A1	06-27-2013	HARVARD COLLEGE [US]		☐

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
/RSN/	001	CHAPIN et al., Rapid microRNA profiling on encoded gel microparticles. Angewandte Chemie International Edition, 50(10):2289-2293, 2011.	☐

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Sheet 2 of 2

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Art Unit	1631
Examiner Name	NEGIN, RUSSELL SCOTT
Attorney Docket Number	38075-716.301

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
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Search Strategy

Databases: BIOSIS Previews®, Embase®, Gale Group Health Periodicals Database, Gale Group PharmaBiomed Business Journals, Gale Group PROMT®, MEDLINE®

Set#	Searched for	Results
S14	encoding and analytes and sample and coding	41°
S13	encoding and analytes and sample and intensity	55°
S12	encoding and analytes and sample and scheme	14°
S11	encoding and analytes and sample	162°
S10	encoding and analytes and degenerate	2°
S9	encoding and analytes and degenrate	0°
S8	encoding and analytes and incremented	0°
S7	encoding and analytes and reagents and incremented	0°
S6	encoding and analytes and reagents and cumulative	9°
S5	encoding and analytes and reagents and presence and absence	70°
S4	encoding and analytes and reagents and presence	102°
S3	encoding and analytes and reagents and binary	14°
S2	encoding and analytes and reagents	132°
S1	encoding and analytes	221°

° Duplicates are removed from the search and from the result count.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14451876	KARTALOV ET AL.
	Examiner	Art Unit
	RUSSELL S NEGIN	1631

CPC- SEARCHED		
Symbol	Date	Examiner
G06F 19/12 with modified terms	11/15/2016	RSN
G06F 19/18 with modified terms	11/15/2016	RSN
G06F 19/34 with modified terms	11/15/2016	RSN
G06F 19/3437 with modified terms	11/15/2016	RSN

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
see attached search history printouts	11/15/2016	RSN
EAST prior art search	11/15/2016	RSN
EAST inventor search on all inventors	11/15/2016	RSN
EAST assignee search	11/15/2016	RSN
PALM inventor search on all inventors	11/15/2016	RSN

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/RUSSELL S NEGIN/ Primary Examiner.Art Unit 1631

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				Filing Date	August 5, 2014
				First Named Inventor	Emil Kartalov
				Art Unit	1631
				Examiner Name	Unassigned
Sheet	1	of	9	Attorney Docket Number	38075-716.301

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	3.	US 2003/0148544 A1	08/07/2003	Nie et al.	
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				Application Number	14/451,876
				Filing Date	August 5, 2014
				First Named Inventor	Emil Kartalov
				Art Unit	1631
				Examiner Name	Unassigned
Sheet	2	of	9	Attorney Docket Number	38075-716.301

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	61.	US 7,101,663	09/05/2006	Godfrey et al.	
	62.	US 7,141,377	11/28/2006	Gelfand et al.	
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	65.	US 7,410,764	08/12/2008	Gocke et al.	
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	69.	US 7,667,024	02/23/2010	Mao et al.	
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	72.	US 7,919,237	04/05/2011	Dimitrov et al.	
	73.	US 7,919,244	04/05/2011	Madejon et al.	
	74.	US 7,941,279	05/10/2011	Hwang et al.	
	75.	US 8,148,512	04/03/2012	Dimitrov	

UNPUBLISHED PATENT APPLICATIONS

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	76.	N/A.			

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/RSN/	77.	EP 1448581 B1	11/12/2008	Dimitrov et al.		
/RSN/	78.	EP 1963531 B1	09/21/2011	Geiss et al.		
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/RSN/	80.	WO 02/056014 A3	10/09/2003	Nelsen et al.		

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/RSN/	81.	WO 2007/076128 A2	07/05/2007	Geiss et al.		
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	85.	WO 2007/076132 A2	07/05/2007	Ferree et al.		
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				Application Number	14/451,876
				Filing Date	August 5, 2014
				First Named Inventor	Emil Kartalov
				Art Unit	1631
				Examiner Name	Unassigned
Sheet	5	of	9	Attorney Docket Number	38075-716.301

NON PATENT LITERATURE DOCUMENTS			
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NON PATENT LITERATURE DOCUMENTS			
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
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L3	1	("20030148544").PN.	US-PGPUB; USPAT; USOCR	OR	OFF	2016/11/17 09:57
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S5	111	rajagopal.in. near aditya.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/15 15:01
S6	0	schere.in. near axel.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/15 15:01
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S17	1234519	encoding	US-PGPUB; USPAT;	OR	OFF	2016/11/15 15:03

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S23	288837	cumulative	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2016/11/15 15:04
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	RUSSELL SCOTT NEGIN
Attorney Docket Number	38075-716.301

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.	Document Number / Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		None			

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No	Foreign Patent Document / Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
/RSN/	001	CN-101570782-A (in Chinese with English abstract)	11-04-2009	CHUNHUA YANG [CN]		x

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]

Examiner Signature	/RUSSELL S NEGIN/	Date Considered	11/15/2016

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 1065
Inventor(s): Kartalov, Emil P., et al.	Examiner: Russell Scott Negin
Application No.: 14/451,876	Art Unit: 1631
Filed: August 5, 2014	Customer No.: 21971
For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE TO OFFICE ACTION

Dear Sir:

This communication is in response to the Non-Final Office Action mailed November 23, 2016. The shortened statutory time period to reply expired on March 23, 2017. Applicants submit this response before May 23, 2017 along with a petition for a two-month time extension and all applicable fees. Applicants believe that this response is timely filed.

Applicants respectfully request reconsideration of the above-referenced patent application in view of the following amendment and remarks.

Amendments to Claims begin on page 2 of this paper.

Remarks begin on page 8 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in this application. The following amendments do not constitute an admission regarding the patentability of the amended subject matter and should not be so construed. Applicants reserve the right to pursue the subject matter of the canceled claims in this or any other appropriate patent application. Further, none of Applicants' amendments are to be construed as dedicating any such subject matter to the public, and Applicants reserve all rights to pursue any such subject matter in this or a related patent application.

Listing of claims:

1. **(Withdrawn)** An assay that is capable of unambiguously detecting the presence or absence of each of at least seven (7) analytes, in any combination of presence or absence, in a single sample volume without immobilization of said analytes, physical separation of said analytes, melting curve analysis, or mass spectrometry.
2. **(Withdrawn)** The assay of claim 1, wherein each of said analytes is encoded as a value of one component of a signal.
3. **(Canceled)**
4. **(Withdrawn)** The assay of claim 2, wherein each of said analytes is encoded as at least one first value in a first component of said signal and at least one second value in a second component of said signal.
5. **(Withdrawn)** The assay of claim 2, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

6.-8. **(Canceled)**

9. **(Withdrawn)** The assay of claim 4, wherein said first value is an intensity or range of intensities and said second value is a wavelength or range of wavelengths.

10.-12. **(Canceled)**

13. **(Withdrawn)** The assay of claim 2, wherein said signal is an electromagnetic signal.

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14.-16. **(Canceled)**

17. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with reagents comprising hybridization probes.

18. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with a plurality of hybridization probes, comprising an identical fluorophore, wherein said plurality of hybridization probes are specific for different analytes.

19. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with a plurality of hybridization probes comprising different fluorophores, wherein said plurality of hybridization probes are specific for different analytes.

20.-21. **(Canceled)**

22. **(Withdrawn)** The assay of claim 2, wherein said signal is generated during a polymerase chain reaction.

23.-26. **(Canceled)**

27. **(Withdrawn)** The assay of claim 4, further comprising constructing a diagram.

28. **(Withdrawn)** The assay of claim 27, wherein said diagram is constructed by plotting possible combinations of said first values and said second values for positive control samples.

29. **(Withdrawn)** The assay of claim 1, wherein at least one of said analytes comprises a polynucleotide.

30.-33. **(Canceled)**

34. **(Withdrawn)** The assay of claim 1, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.

35. **(Withdrawn)** The assay of claim 1, further comprising providing information concerning the presence or absence of said analyte to a health care professional, wherein the health care professional makes a clinical decision based on said information.

36. **(Canceled)**

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37. **(Withdrawn)** The assay of claim 1, wherein at least one step of said assay is performed using a computer.

38. **(Withdrawn)** The assay of claim 37, wherein said computer is located on a remote server.

39. **(Withdrawn)** The assay of claim 37, wherein said computer is located on a thermal cycler.

40.-41. **(Canceled)**

42. **(Withdrawn)** The assay of claim 2, wherein said coding is innately non-degenerate.

43. **(Withdrawn)** The assay of claim 42, wherein said coding is designed to be non-degenerate by enumerating legitimate results that can be obtained from said encoding, identifying legitimate results that are degenerate, and eliminating at least one potential analyte code from said encoding to eliminate degeneracy.

44. **(Canceled)**

45. **(Withdrawn)** The assay of claim 1, wherein said assay is an end-point assay.

46. **(Canceled)**

47. **(Withdrawn)** The assay of claim 1, wherein said assay is a liquid phase assay.

48. **(Canceled)**

49. **(Currently Amended)** A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

    a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by one of said first values, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;
    b. providing a sample comprising, or potentially comprising, at least one of said analytes;
    c. contacting said sample with analyte-specific reagents that generate said first values, as represented in said coding scheme, when each of said analytes is present;
    d. cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and
    e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme;

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wherein said method ~~is capable of~~ non-degenerately detect~~sing~~ the presence or absence of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization of said analytes, physical separation of said analytes, or mass spectometry.

50. **(Canceled)**

51. **(Original)** The method of claim 49, wherein said first value is an intensity or range of intensities.

52. **(Canceled)**

53. **(Previously Presented)** The method of claim 49, wherein said first value is incremented in said coding scheme to an amount greater than the sum of said preceding first values.

54. **(Canceled)**

55. **(Currently Amended)** A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

a. encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

b. providing a sample comprising, or potentially comprising, at least one of said analytes;

c. contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present;

d. cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and

e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme,

wherein~~, when degeneracy is eliminated,~~ said method ~~is capable of~~, without immobilization of said analytes, physical separation of said analytes, or mass

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spectrometry, non-degenerately detects~~ing~~ the presence or absence of each of at least six said analytes in a single sample volume, in any combination of presence or absence, when each of said reagents generates only one second value.

56. **(Original)** The method of claim 55, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

57.-74. **(Canceled)**

75. **(Previously Presented)** The method of claim 49, wherein each of said analytes is encoded as said first value of one component of said signal.

76. **(Previously Presented)** The method of claim 75, wherein each of said analytes is encoded as at least one first value in said first component of said signal and at least one second value in a second component of said signal.

77. **(Previously Presented)** The method of claim 75, wherein each of said analytes is encoded as said first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

78. **(Previously Presented)** The method of claim 76, wherein said first value is an intensity or range of intensities and said second value is a wavelength or range of wavelengths.

79. **(Previously Presented)** The method of claim 49, wherein said signal is an electromagnetic signal.

80. **(Previously Presented)** The method of claim 49, wherein said detecting is performed with reagents comprising hybridization probes.

81. **(Previously Presented)** The method of claim 49, wherein said detecting is performed with a plurality of hybridization probes, comprising an identical fluorophore, wherein said plurality of hybridization probes are specific for different analytes.

82. **(Previously Presented)** The method of claim 49, wherein said detecting is performed with a plurality of hybridization probes comprising different fluorophores, wherein said plurality of hybridization probes are specific for different analytes.

83. **(Previously Presented)** The method of claim 49, wherein said signal is generated during a polymerase chain reaction.

84. **(Previously Presented)** The method of claim 49, further comprising constructing a diagram.

85. **(Previously Presented)** The method of claim 84, wherein said diagram is constructed by plotting possible combinations of said first values and said second values for positive control samples.

86. **(Previously Presented)** The method of claim 49, wherein at least one of said analytes comprises a polynucleotide.

87. **(Previously Presented)** The method of claim 49, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.

88. **(Previously Presented)** The method of claim 49, further comprising providing information concerning the presence or absence of said analyte to a health care professional, wherein the health care professional makes a clinical decision based on said information.

89. **(Canceled)**

90. **(Currently Amended)** The method of claim 49[[89]], wherein said encoding is designed to be non-degenerate by enumerating legitimate results that can be obtained from said encoding, identifying legitimate results that are degenerate, and eliminating at least one potential analyte code from said encoding to eliminate degeneracy.

91. **(Previously Presented)** The method of claim 49, wherein said method does not comprise immobilization of said analytes.

92. **(Previously Presented)** The method of claim 55, wherein said method does not comprise immobilization of said analytes.

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REMARKS

In response to the Non Final Office Action mailed November 23, 2016, Applicants respectfully request consideration of the claims provided herein. With this response, claim 89 is canceled without prejudice or disclaimer.

Claims 49, 51, 53, 55-56, and 75-92 were pending prior to the entry of the above-mentioned amendments. Claim 89 is canceled.

Claims 49, 55 and 90 are amended. Support for amended claims 49 and 55 can be found at least in paragraphs [0003], [0031], [0037], [0039], [0041] and [00119] of the specification as published. Claim 49 has also been amended to correct typographical errors. Claim 90 has been amended to depend from claim 49.

No new matter has been added. Accordingly, claims 49, 51, 53, 55-56, and 75-88 and 90-92 are currently pending.

All amendments to the claims and any cancellations of claims are made without conceding the validity of the rejection; and Applicants reserve the right to pursue any canceled material in any future, related applications.

Claim Rejection-35 U.S.C. § 112 (pre-AIA), second paragraph

Former claim 89 was rejected under U.S.C. § 112 (pre-AIA), second paragraph. *See* Office Action, page 3. In response, without admission of the propriety of this rejection, claim 89 has been cancelled herein and claim 90 has been amended to depend from claim 49. In view of the present cancellation of claim 89, the rejection is rendered moot.

Claim Rejection-35 U.S.C. § 102

Former claims 49, 51, 53, 55, 56, 75-81, 84, 85 and 88-92 were rejected under pre-AIA USC 102(b) as being anticipated by U.S. Patent Application Publication No. 2003/0148544 to Nie et. al. *See* Office Action at p. 5. Claim 89 has been canceled herein.

The Office asserts, *inter alia*, that the capability of "non-degenerately detecting the presence or absence of at least seven analytes in a single sample volume…" as previously recited was interpreted as an "intended use that does not differentiate the recitations of the claims from the prior art".

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In response, without admitting the propriety of the rejection and solely in the interest of advancing prosecution, Applicants have canceled claim 89 and amended independent claims 49 and 55 to recite that the claimed methods are carried out without a step of "immobilization of said analytes, physical separation of said analytes, or mass spectrometry", and has replaced the expression "capable of detecting" with "detects". Applicants respectfully submit that the present amendments to independent claims 49 and 55 render the present rejection moot. However, in the event that a corresponding rejection is raised against any of the presently amended claims, Applicants respectfully traverse such rejection and submit the following argument.

Applicants submit that Nie does not anticipate presently amended independent claims 49 and 55 for at least the following reasons.

Applicant submits that none of the passages of Nie pointed to in the Office Action specifically discloses a method having each and every element of the presently claimed solution. Put another way, none of the cited passages discloses the identical composition "in as complete detail as is contained in the ... claim." *Richardson v. Suzuki Motor Co.*, 868 F.2d 1226, 1236, 9 USPQ2d 1913, 1920 (Fed. Cir. 1989); MPEP 2131. Accordingly, Applicants submit that the present amendments to independent claims 49 and 55 fully address the rejection raised in the Office Action under 35 USC § 102(a)(1).

In particular, Nie fails to teach a method of non-degenerately detecting the presence or absence of at least six (or at least seven) analytes in a single sample volume without immobilization of said analytes, physical separation of said analytes, or mass spectometry.

Applicants respectfully submit that it is evident from the teaching of Nie that the coding methods taught by Nie do not address the issue of degeneracy. Rather, Nie suggests that physical separation of analytes in order to distinguish between identically coded (i.e. degenerately coded) analytes. For example, at paragraph [0084], Nie suggests a method of detecting multiple analytes by flowing conjugates through a microchannel or by spreading on a substrate surface, each of which requires physical separation to allow for resolution of multiple analytes when employing a redundant coding scheme such as that taught by Nie.

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In particular, Applicants submit that Nie explicitly teaches that codes can be formed with *every possible RGB combination*, which, if performed as described in Nie, will necessarily result in a redundancy when attempting to detect six or seven analytes in a single volume, without physical separation, melting curve analysis, or mass spectrometry. For example, based on a review of the potential combinations shown in Figure 1 of Nie, a person of ordinary skill in the art would understand that the codes shown were selected from the set of RGB combinations where the levels for each color channel range, at minimum, from 0 to 10 (hence, 11 values each). Thus, in the embodiment described and depicted in Figure 1, 11x11x11=1331 codes are possible. This is consistent with the following statements set forth in Nie at paragraph [0046]:

> Each color (i.e., wavelength) of the QD can be embedded in the bead at a predetermined intensity, thereby forming a multicolor QD-tagged bead. For each color, the use of 10 intensity levels (0, 1, 2, . . . 9) gives 9 unique codes (10^1-1), because level "0" cannot be differentiated from the background. The number of codes increases exponentially for each intensity and each color used. For example, a three color and 10 intensity scheme yields 999 codes (10^3-1), while a six color and 10 intensity scheme has a theoretical coding capacity of about 1 million (10^6-1). In general, n intensity levels with m colors generate (n^m-1) unique codes. However, the actual coding capabilities are likely to be substantially lower because of spectral overlapping, fluorescence intensity variations, and signal-to-noise requirements. In general, it is more advantageous to use more colors rather than more intensity levels, in order to increase the number of usable codes. The number of intensities is preferably from 0 to 20, more preferably 1-10, more preferably 2-8, more preferably 3-7, more preferably 4-6, more preferably 5-6, and most preferably 6. The number of colors is preferably 1-10 (e.g., 2-8), more preferably, 3-7, and most preferably 5-6. The term "multicolor QD", is meant that the more than one color of luminescent quantum dots are embedded in the bead. Although preferably more than one color of quantum dot is incorporated in the bead, instances wherein one or more colors' intensity is zero, such as a bead with the red:green:blue code of 1:0:0, is also encompassed by the present invention.

For illustrative purposes, three codes selected from this set are as follows:

- Target 1 label is (R=1,G=1,B=1);
- Target 2 label is (R=2, G=2, B=2); and
- Target 3 label is (R=3, G=3, B=3).

With this set, if target 1 is present in isolation, the intensity readout would come from contributions from the Target 1 label only, and would be (1,1,1). Similarly, if target 2 is present in isolation, the readout would be (2,2,2). Likewise, if Target 3 is present in isolation, the readout would be (3,3,3).

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However, if targets 1 and 2 are both present in a measurement, the readout would be (3,3,3): ***the same readout for target 3 in isolation***, and it would be impossible to distinguish between a "target 3 only" signal (3,3,3) and a "target 1 and target 2 both present" signal (1,1,1 and 2,2,2 = 3,3,3). This happens because the codes specified are **degenerate**. Thus, the true combinations of targets cannot be discriminated using the method specified in Nie. Accordingly, Nie does not address this issue of degeneracy, and certainly does not teach or suggest a method that "non-degenerately detects the presence or absence of at least [six or seven] analytes in a single sample volume" as recited by the presently amended independent claims. With respect to the independent claims, these claims all depend from independent claim 49 or 55, and are not anticipated by Nie for at least the same reasons.

For the foregoing reasons, Applicants respectfully request withdrawal of the rejection of claims 49, 51, 53, 55, 56, 75-81, 84, 85, 88 and 90-92, as amended, under pre-AIA 35 U.S.C. 102(b).

Claim Rejection-35 U.S.C. § 103

Former claims 49, 51, 53, 55, 56, 75-81, 84, 85, and 88-92 were rejected under pre-AIA USC 103(a) as being purportedly obvious over Nie further in view Craig, *Nucleic Acids Research*, vol. 18, 1990, pp. 2653-2660, alone or in combination with International Patent Application Publication No. WO/2010/128206 to Orpana et al. *See* Office Action at pp. 9 and 10. Claim 89 has been canceled herein.

No *Prima Facie* Case of Obviousness Established - Not All Limitations Shown

Applicant respectfully traverses the rejection. As discussed above, Nie does not teach or suggest all of the limitations of independent claims 49 or 55, as amended, nor any claim dependent thereon. Further, the teachings of Craig and Orpana, either taken alone or in combination, do not teach or suggest the elements not taught by Nie, for example, these references do not teach a method of non-degenerately detecting the presence or absence of at least six (or at least seven) analytes in a single sample volume without immobilization of said analytes, physical separation of said analytes, or mass spectometry. Further, there is no motivation to modify the teachings of Nie, either alone or in combination, to arrive at the subject

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matter of instant claims. Finally, none of Nie, Craig, nor Orpana, enable the methods recited in the presently amended claims.

Relevant Law

MPEP 2143, states that a *prima facie* case of obviousness may be established only when three basic criteria have been met. "*First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.*"

In Applicants' respectful submission, there is no suggestion or motivation in the teachings of Nie, Craig, or Orpana, either taken alone or in combination, to **non-degenerately detect** the presence or absence of at least six or seven analytes in a single sample volume without immobilization of said analytes, physical separation of said analytes, or mass spectrometry, as recited in independent claims 49 and 55, as amended. Indeed, with reference to the discussion of Nie above regarding the rejections raised under §102, Nie teaches a redundant method, and fails to even address the issue of degeneracy.

Neither Orpana nor Craig cure the defects or Nie. Indeed, neither Orpana nor Craig address the issue of redundancy in a coding scheme as recited in the presently amended claims.

Regarding the methods taught by Craig, these methods require spotting analytes onto nylon membranes. *See* Craig at p. 2654, first column. Accordingly, the method taught by Craig does not teach detection of the "presence or absence of at least six [or seven] analytes in a ***single sample volume***... ***without immobilization of said analytes or physical separation*** of said analytes". Indeed, the methods taught by Craig clearly require both immobilization and physical separation (on the nylon membranes), in order to allow for resolution and combination of the results obtained for each of the various probes. *See*, *e.g.*, Craig, Fig. 2.

Regarding the methods taught by Orpana, these methods fail to provide any teaching or suggestion of any method of coding, nor do they address the issue of coding or degeneracy. In particular, Orpana fails to cure the defects of Nie and Craig discussed above, and fails to teach or suggest any method of encoding and non-degenerately detecting the presence or absence of at

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least six or seven analytes in a single sample volume without immobilization of said analytes, physical separation of said analytes, or mass spectrometry, as recited in the amended independent claims 49 and 55.

Conclusion

Accordingly, Applicants submits that the test set out at MPEP 2143 is not met in the present case, that the cited references, either taken alone or in combination, fail to render obvious the subject matter claimed by independent claims 49 and 55, as amended, and that a *prima facie* case of obviousness has not been established in the present case for those independent claims or the claims that depend therefrom. Favorable consideration of the amended claims is respectfully requested.

Claim Rejection-35 U.S.C. § 101

The Office had rejected former claims 49, 51, 53, 55, 56, 75-81 and 83-92 as being allegedly directed to a judicial exception "without significantly more". *See* Office Action at page 4. The Office went on to allege that the rejected claims were "directed to the abstract idea of encoding signals in a manner that reduces or eliminates degeneracy," and that the claims did "not include additional elements that [were] sufficient to amount to significantly more than the judicial exception… because providing samples, performing assays, and determin[ing] presence/absence patterns of analytes is routine and conventional in the prior art". *Id.*

In response, without admitting the propriety of the rejection and solely in the interest of advancing prosecution, Applicants have canceled claim 89 and amended independent claims 49 and 55 to recite that the claimed methods do not require "immobilization of said analytes, physical separation of said analytes, or mass spectrometry". Applicants respectfully submit that the present amendments to independent claims 49 and 55 render the present rejection moot. However, in the event that a corresponding rejection is raised against any of the presently amended claims, Applicants respectfully traverse such rejection and submit the following argument.

A. The Present Rejection Fails to Account for the Specific Requirements of the Claims

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In the *Alice* decision, the Supreme Court applied a two-step framework for determination of patent eligibility under Section 101. First, one must "determine whether the claims at issue are directed to a patent-eligible concept." *Alice* at 2355. Second, one "must examine the elements of the claims to determine whether it contains an inventive concept sufficient to transform the claimed abstract idea into a patent-eligible application." *Id.* at 2357. In the second step, the claim elements must be analyzed separately and as an "ordered combination." *Id.* at 2359.

On November 2, 2016, the Robert W. Bahr, the Deputy Commissioner for Patent Examination Policy, issued a memorandum regarding "Recent Subject Matter Eligibility Decisions" (hereinafter "Nov. 2016 Eligibility Memo"). The Nov. 2016 Eligibility Memo is intended provide guidance to examiners "about finding eligibility for software claims." The Nov. 2016 Eligibility Memo at page 1. Here, the Nov. 2016 Eligibility Memo "provides a discussion of two of the recent decisions identifying eligible subject matter, namely *McRO, Inc. dba Planet Blue v. Bandai Namco Games America Inc.*, 120 USPQ2d 1091 (Fed. Cir. 2016) and *BASCOM Global Internet Services v. AT&T Mobility LLC*, 827 F.3d 1341 (Fed. Cir. 2016). *Id.* Specifically, the Nov. 2016 Eligibility Memo noted:

- "The *McRO* court cautioned that courts '**must be careful to avoid oversimplifying the claims**' by looking at them generally and **failing to account for the specific requirements of the claims**." *Id.* at page 2 (emphasis added).
- Following the section titled "Notable Points from *McRO*," the Nov. 2016 Eligibility Memo stated how "[e]xaminers should consider the claim **as a whole** under Step 2A of the USPTO's SME guidance, and **should not overgeneralize the claim or simplify it into its "gist" or core principles**, when identifying a concept as a judicial exception." *Id.* (emphasis added).
- Notwithstanding the claims having "additional elements [that] were generic computer, network, and Internet components that did not amount to significantly more when considered individually," the *BASCOM* court still "recognize[d] that **when combined, an inventive concept may be found in the non-conventional and non-generic arrangement**." *Id.* at page 3.

- Following the section titled "Notable Points from *BASCOM*," the Nov. 2016 Eligibility Memo stated how "examiners should – when analyzing Step 2B of the USPTO's SME guidance – consider the additional elements in **combination**, as well as individually, when determining whether a claim as a whole amounts to significantly more, as this may be found in the non-convention and non-generic arrangement of known, conventional elements." *Id.* (emphasis in original).

As set out above, the Office alleges that the rejected claims were "directed to the abstract idea of encoding signals in a manner that reduces or eliminates degeneracy," and that the claims did "not include additional elements that [were] sufficient to amount to significantly more than the judicial exception… because **providing samples, performing assays, and determin[ing] presence/absence patterns of analytes is routine and conventional in the prior art**". Office Action at p. 4 [emphasis added].

However, in so doing, the Office fails to account for the claims' **specific** requirements – a failure the Nov. 2016 Eligibility Memo specifically warns against. *See* the Nov. 2016 Eligibility Memo at 2.

By way of example, claim 49 recites:

> **A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising**:
>
> a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by one of said first values, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;
> b. **providing a sample comprising, or potentially comprising, at least one of said analytes**;
> c. **contacting said sample with analyte-specific reagents** that generate said first values, as represented in said coding scheme, when each of said analytes is present;
> d. **cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement**; and
> e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme;
> **wherein said method is non-degenerately detects the presence or absence of at** least **seven analytes in a single sample volume, in any combination of presence or absence, without immobilization of said analytes, physical separation of said analytes, or mass spectometry**. [Emphasis to exemplary specific elements added].

Rather than addressing the specific elements recited in the claim, as presently amended, including the exemplary specific elements highlighted above, the Office generalizes the entire claim with a broad description. In doing so, the Office simplifies the claim to its "gist" or core principles. This is improper. The Nov. 2016 Eligibility Memo specifically prohibits this. The Nov. 2016 Eligibility Memo at page 2 ("Examiners should consider the claim as a whole under Step 2A of the USPTO's SME guidance, and should not overgeneralize the claim or simplify it into its 'gist' or core principles, when identifying a concept as a judicial exception.").

To summarize, the Office oversimplifies the claims, and fails to address the specific elements recited in the claims. By generalizing the claims (rather than accounting for the specific requirements of the claims), the Office fails to adhere to its own guidelines in the Nov. 2016 Eligibility Memo. For at least these reasons, Applicant disagrees that the Office has properly satisfied the first step and respectfully requests that the Office withdraw the present rejection.

B. The Claims Recite an Inventive Concept

Even assuming, *arguendo*, that independent claims 49 and 55 are directed to an abstract idea, which is traversed in Sections II.A and II.B, *supra*, the claims recite "significantly more" than an abstract idea itself.

The Nov. 2016 Eligibility Memo provides guidance on how examiners should apply the second step. *See* the Nov. 2016 Eligibility Memo at page 3. Specifically, the Nov. 2016 Eligibility Memo explicated the *BASCOM* case, highlighting how the *BASCOM* court found an inventive concept in the "claimed system for filtering content retrieve from an Internet computer network" may be found notwithstanding additional elements being "generic computer, network, and Internet components." *Id.*

A closer examination of *BASCOM* provides further guidance on how to apply the second step. In *BASCOM*, the lower court applied the second step by looking at each limitation individually and collectively and finding that there was no inventive concept because "the limitations, 'considered individually, or as an ordered combination, are no more than routine additional steps involving generic computer components and the Internet, which interact in well-known ways to accomplish the abstract idea of filtering Internet content." *BASCOM*, 827 F.3d at 1349.

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The Federal Circuit held the lower court erred in its analysis – in particular its conclusion about the lack of inventive concept as it pertained to "the ordered combination of limitations." *Id.* The Federal Circuit emphasized that the "inventive concept inquiry requires more than recognizing that each claim element, by itself, was known in the art" and noted how the lower court's analysis improperly "looked similar to an obviousness analysis under 35 U.S.C. § 103, except lacking an explanation of a reason to combine the limitations as claimed." *Id.* at 1350. The Federal Circuit then proceeded to describe the inventive concept of the relevant patent by pointing to specific parts of the specification and ultimately found that the "specific method of filtering Internet content cannot be said, as a matter of law, to have been conventional or generic":

> The inventive concept described and claimed in the [] patent is the installation of a filtering tool at a specific location, remote from the end-users, with customizable filtering features specific to each end user. This design gives the filtering tool both the benefits of a filter on a local computer and the benefits of a filter on the ISP server. BASCOM explains that the inventive concept rests on taking advantage of the ability of at least some ISPs to identify individual accounts that communicate with the ISP server and to associate a request for Internet content with a specific individual account. According to BASCOM, the inventive concept harnesses this technical feature of networking technology in a filtering system by associating individual accounts with their own filtering scheme and elements while locating the filtering system on an ISP server.

Id. (internal citations to patent specification and oral arguments omitted).

The facts and holding in *BASCOM* are analogous to the present situation.

First, like the overturned lower court in *BASCOM*, the Office erred in its analysis by arguing how the recitation of "providing samples, performing assays, and determin[ing] presence/absence patterns of analytes is routine and conventional in the prior art"." Office Action at p. 4. Thus, the Office's argument amounts to an obviousness analysis under 35 U.S.C. § 103 without the explanation of a reason to combine the limitations as claimed – an argument the Federal Circuit in *BASCOM* specifically held was improper in the second step. *BASCOM*, 827 F.3d at 1350.

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Next, like the specific method of filtering Internet content in *BASCOM*, a very specific system, namely analytes in a sample volume, and method of detecting the presence or absence of those analytes in that single sample volume **(without immobilization of said analytes, physical separation of said analytes, or mass spectrometry)** exists in the presently claimed methods. In other words, the present claims have specific applications in the marketplace, which "are not likely to be so abstract that they override the statutory language and framework of the Patent Act." *See Res. Corp. Techs. v. Microsoft Corp.*, 627 F.3d 859, 869 (Fed. Cir. 2010).

Finally, the claims in *BASCOM* sought to overcome the technical challenges associated with networking technology with a filtering system by associating individual accounts with their own filtering scheme and elements while locating the filtering system on an ISP server. *See BASCOM*, 827 F.3d at 1350. Similarly, the claims at issue seek to overcome the technical challenges associated with detecting the presence or absence of six or seven analytes in a single volume namely difficulties associated with detection of such analytes in that single sample volume without immobilization of said analytes, physical separation of said analytes, or mass spectrometry, as discussed further above with respect to the rejections under 35 USC §§ 102 and 103.

In sum, the second step analysis, when conducted in a manner consistent with the governing caselaw and the Nov. 2016 Eligibility Memo, should arrive at a conclusion that the presently amended claims recite an inventive concept that renders the claims patent eligible under Section 101. Under *BASCOM* and the Office's own guidelines set forth in the Nov. 2016 Eligibility Memo, the independent claims 49 and 55 in the pending application recite an inventive concept and satisfy the second step. For at least these reasons, Applicants submit that the second step is satisfied and respectfully request the Office to consider the claims as being patent eligible under Section 101, and to withdraw the rejection.

CONCLUSION

Applicants believe that the application is in condition for allowance and respectfully solicit the Examiner to expedite prosecution of this patent application to issuance. Should the Examiner have any questions, the Examiner is encouraged to telephone the undersigned.

The Commissioner is authorized to charge any additional fees which may be required, including petition fees and extension of time fees, to Deposit Account No. 23-2415 (Docket No. 38075-716.301).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: May 1, 2017 By: /Lee A. Johnson/

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Electronic Patent Application Fee Transmittal	
Application Number:	14451876
Filing Date:	05-Aug-2014
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Filer:	Vernon A. Norviel/Katherine Lopez
Attorney Docket Number:	38075-716.301
Filed as Small Entity	
Filing Fees for Utility under 35 USC 111(a)	

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 2 months with $0 paid	2252	1	300	300
Miscellaneous:				
		Total in USD ($)		**300**

Electronic Acknowledgement Receipt	
EFS ID:	29088549
Application Number:	14451876
International Application Number:	
Confirmation Number:	1065
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Customer Number:	21971
Filer:	Vernon A. Norviel/Katherine Lopez
Filer Authorized By:	Vernon A. Norviel
Attorney Docket Number:	38075-716.301
Receipt Date:	02-MAY-2017
Filing Date:	05-AUG-2014
Time Stamp:	11:29:20
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1		CIT_38075_716301_Resp_OA.pdf	280209 3cc108082e35e7d7cafb47dac2147ce7bc82d12a	yes	19

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	7
	Applicant Arguments/Remarks Made in an Amendment	8	19

Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30864 fb92d19035f668708ec669d127d49aa81444eb38	no	2
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National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

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(Use as many sheets as necessary)

Sheet 1 of 5

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	RUSSELL SCOTT NEGIN
Attorney Docket Number	38075-716.301

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	001	US-7930106	04-19-2011	CARRICK; James M.	
	002	US-8039215	10-18-2011	HIGUCHI; Russell et al.	
	003	US-8426132	04-23-2013	LI; Bin et al.	
	004	US-8455184	06-04-2013	ATCHLEY; Daniel H. et al.	
	005	US-8492094	07-23-2013	DIMITROV; Krassen et al.	
	006	US-8519115	08-27-2013	WEBSTER; Philippa J. et al.	
	007	US-8614061	12-24-2013	BRABETZ; Werner et al.	
	008	US-8771955	07-08-2014	REED; Michael W. et al.	
	009	US-8838394	09-16-2014	KARTALOV; Emil et al.	
	010	US-8877464	11-04-2014	BABIEL; Reiner et al.	
	011	US-8962250	02-24-2015	STANLEY; Keith	
	012	US-9133506	09-15-2015	KATZIR; Nir et al.	
	013	US-9222128	12-29-2015	SAXONOV; Serge et al.	
	014	US-9260761	02-16-2016	TYAGI; Sanjay et al.	
	015	US-9422593	08-23-2016	ROTHMANN; Thomas et al.	
	016	US-9447457	09-20-2016	CHUN; Jong Yoon et al.	
	017	US-20130017971	01-17-2013	GEISS; Gary K. et al.	
	018	US-20130261019	10-03-2013	LIN; Chenxiang et al.	
	019	US-20140038195	02-06-2014	MALIK; Imran R. et al.	
	020	US-20150140554	05-21-2015	SNYDER; Richard O. et al.	

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Application Number	14451876
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First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	RUSSELL SCOTT NEGIN
Attorney Docket Number	38075-716.301

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	001	EP-1629108-B1	12-31-2014	PHRI PROPERTIES INC [US]		☐
	002	WO-2004087950-A2	10-14-2004	ROCHE DIAGNOSTICS GMBH [DE], et al.		☐

Examiner Signature		Date Considered	

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Sheet 3 of 5

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Attorney Docket Number	38075-716.301

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Sheet 4 of 5

Complete if Known	
Application Number	14451876
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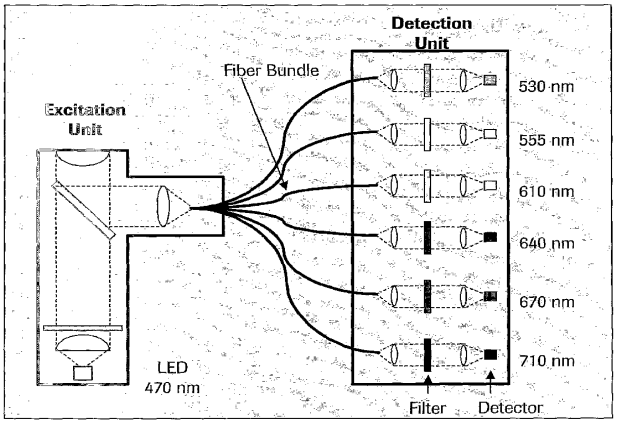
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[Continued on next page]

(54) Title: IMPROVED SYSTEM FOR MULTI COLOR REAL TIME PCR



(57) Abstract: The invention is directed to a system for performing multi-color real time PCT, comprising a flexible real time PCT instrument and a specific composition or reaction mixture for performing multiplex PCR: In particular, the present invention is directed to a composition or reaction mixtuer which comprises at least 3, preferably 4-5 and most preferably exactly 4 pairs of FRET hybridization probes. Each pair of said hybridization probes consists of a FRET donor probe carrying a FRET donor moiety and a FRET acceptor probe carrying a FRET acceptor moiety having an emission maximum between 550 and 710 nm.

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Improved system for multi color real time PCR

Field of invention

The present invention relates to the field of Real time PCR. In particular, the present invention is directed to a system for performing multiplex real time PCR.

Prior art background

Amplification of DNA by polymerase chain reaction (PCR) is a technique fundamental to molecular biology. Nucleic acid analysis by PCR requires sample preparation, amplification, and product analysis. Although these steps are usually performed sequentially, amplification and analysis can occur simultaneously. DNA dyes or fluorescent probes can be added to the PCR mixture before amplification and used to analyze PCR products during amplification. Sample analysis occurs concurrently with amplification in the same tube within the same instrument. This combined approach decreases sample handling, saves time, and greatly reduces the risk of product contamination for subsequent reactions, as there is no need to remove the samples from their closed containers for further analysis. The concept of combining amplification with product analysis has become known as "real time" PCR. See, for example, U.S. Patent No. 6,174,670.

Monitoring fluorescence during each cycle of PCR initially involved the use of ethidium bromide. (Higuchi R, G Dollinger, PS Walsh and R. Griffith, Simultaneous amplification and detection of specific DNA sequences, Bio/Technology 10 (1992) 413-417; Higuchi R, C Fockler G Dollinger and R Watson, Kinetic PCR analysis: real time monitoring of DNA amplification reactions, Bio/Technology 11 (1993) 1026-1030). In that system fluorescence is measured once per cycle as a relative measure of product concentration. Ethidium bromide detects double stranded DNA; if template is present fluorescence intensity increases with temperature cycling. Furthermore, the cycle number where an increase in fluorescence is first detected increases inversely proportionally to the log of the initial template concentration. Other fluorescent systems have been developed that are capable of providing additional data concerning the nucleic acid concentration and sequence.

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In kinetic real time PCR, the formation of PCR products is monitored in each cycle of the PCR. The amplification is usually measured in thermocyclers which have additional devices for measuring fluorescence signals during the amplification reaction.

Real time PCR instrumentation

Several types of real time detection thermocyclers are known in the art.

EP 0 640 828, for example discloses an apparatus for monitoring multiple nucleic acid amplification simultaneously. It is characterized in that it comprises a metal block thermal cycler, including a heat conducting member having multiple recesses formed therein in order to take up a multiwell plate such as a microtiter plate. Detection is obtained by means of a CCD camera, arranged for detecting light emitted (simultaneously) from all of said recesses. Alternatively, the use of fiber optics is suggested. Depending on the fluorescent dyes which are used and – more important-, depending on the presence of a filter wheel close to the CCD camera, the system has the capability of analyzing multiplex amplification reactions, wherein in one reaction chamber, one or more different amplicons are detected by 2 or more differently labeled hybridization probes. Yet, EP 0 640 828 does neither anticipate nor suggest, which labels or detection formates could be used for such a multiplex/multicolor approach.

US 6,015,674 discloses an apparatus and a system for real time PCR detection and quantification, characterized in that it is capable of detecting first and second fluorescent indicators, which may be used as labels for different hybridization probes in order to detect different amplicons in the same reaction vessel. Yet, US 6,015,674 does not disclose a system for performing multipex experiments with a higher degree of complexity.

Another typical example is the Roche Diagnostics LightCycler (Cat. No. 2 0110468). It is a fast PCR system enabling kinetic on-line PCR quantification and subsequent analysis of PCR-product melting curves. The optical system of the current LightCycler version 1.2 being commercially available contains one light source, a blue light emitting diode (470 nm LED) and three detection channels. The amplification products are detected by means of fluorescent labeled hybridization

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probes which only emit fluorescence signals when they are bound to the target nucleic acid or in certain cases also by means of fluorescent dyes that bind to double-stranded DNA. A defined signal threshold is determined for all reactions to be analysed and the number of cycles Cp required to reach this threshold value is determined for the target nucleic acid as well as for the reference nucleic acids such as the standard or housekeeping gene. The absolute or relative copy numbers of the target molecule can be determined on the basis of the Cp values obtained for the target nucleic acid and the reference nucleic acid.

The fluorescence emitted by a sample is separated by a set of dichroic mirrors and filters into different wavelengths that can be recorded in one of the three detection channels (530 / 640 / 710 nm). This allows detection of the double-stranded DNA-binding dye SybrGreenI, mono color detection of the TaqMan Probe format and dual color detection of the Hybridization Probe(HybProbe) format. Details of the Lightcycler system are disclosed in WO 97/46707, WO 97/46712 and WO 98/46714, The complete contents of these applications are herewith incorporated by reference.

A very important feature of the LightCycler instrument is the color compensation software. In principle this software allows for accurate quantification and melting curve analysis by means of correcting spectral overlap of monitored fluorescent radiation in a temperature dependent manner. Technical details are disclosed in US 6,197,520.

Similar to the LightCycler system, the Corbett Rotor-Gene Real time PCR Thermocycler (www.corbettresearch.com) is a 4 channel multiplexing system comprising 4 different LEDs as excitation sources and corresponding photodiodes as fluorescent detection units. Thus, although this instrument hardware at least theoretically has the capacity of performing multiplex experiments with up to four differently labeled hybridization probes within one reaction vessel, no respective successful application protocol has been published so far.

Another real time PCR instrument ist the Biorad iQ Multi-color Real time PCR detection system (Cat. No: 170-8740), which allows for a fluorophore excitation and emission from 400 nm to 700 nm. The system is based on a conventional multiwell heating block for thermocycling, a tungsten lamp as an excitation source, a filter wheel for providing appropriate excitation wavelengths, a second filter wheel

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for selecting appropriate emission wavelengths and a CCD camera as a detection unit. The instrument has successfully been used in a multiplex assay for the detection of 4 different amplicons generated from targets with more or less equimolar concentrations, using four differently labeled TaqMan probes in the same reaction vessel (Pedersen, S., Bioradiations 107 (2001) 10-11).

In a further approach to increase multiplexing capacities, US 6,369,893 discloses a real time PCR thermocycling instrument comprising a first optics assembly with at least two light sources and a second optics assembly comprising at least two detectors for detecting and discriminating light of different emission wavelengths. In particular, a specific embodiment of different 4 LEDs as light sources and 4 different photodiodes as detectors is disclosed. Thus, the instrument disclosed in 6,369,893 in principle can be used for real time PCR detection with a broad selection of different fluorescent dyes which are known in the art. Yet, US 6,369,893 does neither anticipate nor suggest any approach on how a multiplex experiment comprising multiple different probes each labeled with a different fluorescent entity needs to be designed.

Real time PCR detection formates

In general, there exist different formates for real time detection of amplified DNA, of which the following are well known and commonly used in the art:

a) **DNA binding dye formate**

Since the amount of double stranded amplification product usually exceeds the amount of nucleic acid originally present in the sample to be analyzed, double-stranded DNA specific dyes may be used, which upon excitation with an appropriate wavelength show enhanced fluorescence only if they are bound to double-stranded DNA. Preferably, only those dyes may be used which like SybrGreenI I, for example, do not affect the efficiency of the PCR reaction.

All other fomates known in the art require the design of a fluorescent labeled Hybridization Probe which only emits fluorescence upon binding to its target nucleic acid.

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b) TaqMan probe

A single-stranded Hybridization Probe is labeled with two components. When the first component is excited with light of a suitable wavelength, the absorbed energy is transferred to the second component, the so-called quencher, according to the principle of fluorescence resonance energy transfer. During the annealing step of the PCR reaction, the hybridization probe binds to the target DNA and is degraded by the 5'-3' exonuclease activity of the Taq Polymerase during the subsequent elongation phase. As a result the excited fluorescent component and the quencher are spatially separated from one another and thus a fluorescence emission of the first component can be measured (US5,538,848).

c) Molecular Beacons

These hybridization probes are also labeled with a first component and with a quencher, the labels preferably being located at both ends of the probe. As a result of the secondary structure of the probe, both components are in spatial vicinity in solution. After hybridization to the target nucleic acids both components are separated from one another such that after excitation with light of a suitable wavelength the fluorescence emission of the first component can be measured (US 5,118,801).

d) Single Label Probe (SLP) Format

This detection format consists of a single oligonucleotide labeled with a single fluorescent dye at either the 5'- or 3'-end (WO 02/14555). Two different designs can be used for oligo labeling: G-Quenching Probes and Nitroindole-Dequenching probes.

In the G-Quenching embodiment, the fluorescent dye is attached to a C at oligo 5'- or 3'-end. Fluorescence decreases significantly when the probe is hybridized to the target, in case two G's are located on the target strand opposite to C and in position 1 aside of complementary oligonucleotide probe.

In the Nitroindole Dequenching embodiment, the fluorescent dye is attached to Nitroindole at the 5'- or 3'-end of the oligonucleotide. Nitroindole somehow

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decreases the fluorescent signaling of the free probe. Fluorescence increases when the probe is hybridized to the target DNA due to a dequenching effect.

e) FRET hybridization probes

The FRET Hybridization Probe test format is especially useful for all kinds of homogenous hybridization assays (Matthews, J.A., and Kricka, L.J. , Analytical Biochemistry 169 (1988) 1-25. It is characterized by a pair of two single-stranded hybridization probes which are used simultaneously and are complementary to adjacent sites of the same strand of the amplified target nucleic acid. Both probes are labeled with different fluorescent components. When excited with light of a suitable wavelength, a first component transfers the absorbed energy to the second component according to the principle of fluorescence resonance energy transfer such that a fluorescence emission of the second component can be measured when both hybridization probes bind to adjacent positions of the target molecule to be detected.

When annealed to the target sequence, the hybridization probes must sit very close to each other, in a head to tail arrangement. Usually, the gap between the labeled 3' end of the first probe and the labeled 5' end or the second probe is as small as possible, i.e.1-5 bases. This allows for a close vicinity of the FRET donor compound and the FRET acceptor compound, which is typically 10-100 Angstroem.

Alternatively to monitoring the increase in fluorescence of the FRET acceptor component, it is also possible to monitor fluorescence decrease of the FRET donor component as a quantitative measurement of hybridization event.

In particular, the FRET Hybridization Probe format may be used in real time PCR, in order to detect the amplified target DNA. Among all detection formats known in the art of real time PCR, the FRET-Hybridization Probeformat has been proven to be highly sensitive, exact and reliable (WO 97/46707; WO 97/46712; WO 97/46714). Yet, the design of appropriate FRET Hybridization Probe sequences may sometimes be limited by the special characteristics of the target nucleic acid sequence to be detected.

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As an alternative to the usage of two FRET hybridization probes, it is also possible to use a fluorescent-labeled primer and only one labeled oligonucleotide probe (Bernard, P. S., et al., Analytical Biochemistry 255 (1998) 101-107). In this regard, it may be chosen arbitrarily, whether the primer is labeled with the FRET donor or the FRET acceptor compound.

Besides PCR and real time PCR, FRET hybridization probes are used for melting curve analysis. In such an assay, the target nucleic acid is amplified first in a typical PCR reaction with suitable amplification primers. The hybridization probes may already be present during the amplification reaction or added subsequently. After completion of the PCR-reaction, the temperature of the sample is constitutively increased, and fluorescence is detected as long as the hybridization probe was bound to the target DNA. At melting temperature, the hybridization probes are released from their target, and the fluorescent signal is decreasing immediately down to the background level. This decrease is monitored with an appropriate fluorescence versus temperature-time plot such that a first derivative value can be determined, at which the maximum of fluorescence decrease is observed.

There exist many different pairs of fluorescent dyes known in the art which according to the invention are principally capable of acting together as a FRET donor/FRET acceptor pair. Yet, prior to the present invention, no functional example has been disclosed, characterized in that 4 different FRET pairs have succesfully been used in a multiplex detection assay. Among other reasons, this may be due to lack of approriate instrumentation and, moreover, due to fact that the functionality of the FRET process of a specific FRET pair is interfered by other fluorecent compounds which are present in the same reaction mixture.

As discussed above, there exist different real time detection thermocycler instruments having a maximum of 4 detector channels for multiplex/multicolor detection. Yet, the utility of all of these instruments for multiplex detection up to now has been very limited due to the fact that attempts to establish real time multicolor multiplex assays with several (at least more than two) differently labeled probes with sufficient sensitivity and specificity have not been successful so far.

Thus, it was the object of the present invention to provide an improved system which allows for an optimized and at the same time flexible design of

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multiplex/multicolor detection experiments. In one aspect the problem to be solved relates to improvements in the design of approriate hybridization probes. In another aspect, the problem to be solved relates to an improvement of instrumentation.

Brief description of the invention:

Thus, the new invention is directed to a real time PCR system with an extension of applicable detection formats and multicolor features. The system comprises specific reagent mixtures for multiplex/multicolor detection as well as an improved real time PCR instrument with an optical detection system comprising multiple detection units. The optical system is designed to provide excitation sources and detection channels for multiple detection formats, for example: SybrGreenI, multiplex FRET-Hybridization Probes, and multiplex TaqMan probes.

More precisely, the invention is directed to a system for performing multi-color real time PCR, comprising a real time PCR instrument and a specific composition or reaction mixture for performing multiplex PCR:

The composition or reaction mixture of the present invention comprises at least 3, preferably 4-5 and most preferably exactly 4 pairs of FRET hybridization probes. Each pair of said hybridization probes consists of a FRET donor probe carrying a FRET donor moiety and a FRET acceptor probe carrying a FRET acceptor moiety having an emission maximum between 550 and 710 nm.

In one embodiment at least 3, preferably at least 4 and most preferably exactly 4 FRET donor moieties are identical. Most preferably, all FRET donor moieties are identical. In other words, if all FRET donor moieties of the different FRET pairs are identical, they can be excited with the same excitation source.

In another embodiment of the invention at least 3, preferably at least 4 and most preferably exactly 4 FRET donor moieties are Fluorescein. Most preferably, all FRET donor moieties are Fluorescein.

In a specific embodiment, at least one additional FRET donor moiety is selected from a group consisiting of Atto425 and WI343.

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In another specific embodiment, which is not at all mutual exclusive to those embodiments disclosed above, one FRET acceptor moiety is selected from a group consisting of LC-Red 705 , Cy5.5, JA286 and a sulfonated cyanine dye as disclosed in US 6,027,709, formula 1.

As a second FRET acceptor moiety Cy5 may be selected.

As a third FRET acceptor moiety, LC-Red 640 may be used.

As a fourth FRET acceptor moiety, the LCRed derivative LC-Red 610 is chosen.

In a particular embodiment, the fifth FRET acceptor moiety is selected from a group consisting of Rh6G and TAMRA.

The instrument part of the system is characterized in that said real time PCR instrument comprises

- at least 1 light source, preferably an LED
- at least 4 and preferably 5-6 fluorescent detector entities, each of said entities having central detection wavelengths which are distinct from each other by at least 25 and preferably at least 30 nm

characterized in that said detector entities are capable of

- simultaneously detecting maximum fluorescene emission of at least 3, preferably 4 and most preferably 5 differently labeled FRET Hybridization Probe pairs,
- simultaneously detecting maximum fluorescence emission of at least 2 differently labeled TaqMan hybridization probes, and
  - detecting maximum fluorescence emission of SybrGreenI
  - means for heating and cooling

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- multiple reaction vessels for containing a reaction mixture.

In a prefered embodiment of the invention the instrument part of the system is characterized in that said real time PCR instrument comprises exactly one light source.

In this context it is understood that although the instrument is being capable of detecting fluorescence emission of the FRET format, the TaqMan format and the SybrGreen format, the term „simultaneously" means that within the same thermcycling protocol (i. e. within one run of the instrument), either at least 3 differently labeled FRET Hybridization Probes or at least 2 differently TaqMan probes or SybrGreenI are detected.

Preferably, the instrument comprises at least 24, more preferably 32 and most preferably 48 of said reaction vessels.

Also preferably, said centrally detected wavelengths are selected from a group of range of wavelengths, said group consisting of 520-540 nm, 545-565 nm, 570-590 nm, 600-620 nm, 630-650 nm, 660-680 nm, and 700-720 nm.

Also preferably, each reaction vessel is characterized in that fluorescence excitation by said fluorescent light sources and fluorescence monitoring by said fluorescent detector entities are obtained along the same axis of said reaction vessel.

It has been proven to be particular advantageous, if said light sources and said detector entities are located in separate housings.

It has also been proven to be particular advantageous, if said means for heating and cooling are means of forced liquid or forced gas, preferentially means of forced air.

It has further been proven to be particular advantageous, if the reaction vessels are fixed in a rotating carousel.

In a last aspect, the invention is also directed to a corresponding method for amplyfying and detecting multiple target DNA sequences comprising

a) providing a reaction mixture as disclosed above,

b) subjecting said reaction mixture to a thermoclyling protiocol such that amplification of said multiple target sequences can take place

c) monitoring hybridization of each of said pairs of FRET hybridization probes at least once after a plurality of amplification cycles.

Hybridization may also be monitored at least once in a temperature dependent manner in order to perform a melting curve analysis.

Detailed description of the invention:

A) **Instrument**

In a first aspect, a system according to the invention comprises an instrument suitable for real time PCR. Optionally, the instrument is also suited for melting curve analysis, i. e. monitoring temperature dependent binding of a DNA binding entity such as a hybridization probe or a ds DNA binding dye.

The instrument basically consists of an optical part and a means for thermocycling which can subject multiple amplification solutions in their respective reaction vessels to a process of repeated thermocycling such that a polymerase chain reaction (PCR) can take place.

Preferably, the instrument part of the system is characterized in that said real time PCR instrument comprises

- at least 1 light source, preferably a light emitting diode
- at least 4 and preferably 5-6 fluorescent detector entities, each of said entities having central detection wavelengths which are distinct from each other by at least 25 and preferably at least 30 nm

characterized in that said detector entities are capable of

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- simultaneously detecting maximum fluorescene emission of at least 3, preferably 4 and most preferably 5 differently labeled FRET Hybridization Probe pairs,

- simultaneously detecting maximum fluorescence emission of at least 2 differently labeled TaqMan hybridization probes, and

- detecting maximum fluorescence emission of SybrGreenI

- means for heating and cooling, and

- multiple reaction vessels for containing a reaction mixture.

Most preferably, the instrument part of the system is characterized in that said real time PCR instrument comprises exactly one light source.

The means for thermocycling can be chosen arbitrarily. For example, metal block cyclers which are well known in the art may be used. Yet it is advantageous if the means for thermocycling provide means for active heating as well as independent means for active cooling. Moreover it has also been proven to be particular advantageous, if said means for heating and cooling are means of forced liquid or forced gas. In a specific embodiments said means use forced air.

It is also prefered, if said means for thermocycling are capable of performing rapid thermocycling like it is the case in the LightCycler technology (WO 97/46712). As it can be deduced from WO 97/46712, this technology is based on a forced air heating characterized in that ambient air is heated by a heating coil and forced air cooling characterized in that ambient air is provided.

A further improvement with respect to rapid thermocycling is the usage of capillaries as reaction vessels due to there comparatively high surface/volume ratio. Capillaries with an outer diameter of less than 5 mm, preferably of less than 3,2 mm and most preferably of less than 1,6 mm have been proven by the inventors to be particular advantageous, since they allow for reaction mixtures having total volumes between 10 and 100 µl which still can be subjected to a rapid thermocycling protocol with a reaction time of less than 2 minutes per cycle.

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Moreover, each reaction vessel needs to be fabricated at least partially or completely of an optically clear material such as glas or plastic.

Positioning of each reaction vessel (capillary) in a monitoring position is preferably characterized in that fluorescence excitation and monitoring are performed along the same axis of said reaction vessel. Such a principle enhances signalling due the phemomenon of total internal reflectance.

It has further been proven to be particular advantageous, if the reaction vessels are fixed in a rotating carousel in order to generate uniform thermocycling profiles throughout the multiple reaction vessels.

The optical part of the instrument basically consists of an excitation unit with one or more appropriate light sources in order to excite the fluorescent compound(s) which are present in the reaction solutions and a detection unit for detecting fluorescence emission from said reaction vessels.

According to WO 97/46712, it is possible to construct an optical system wherein the excitation unit and the detection unit are placed in a common housing, provided that an appropriate number of dichroidic mirrors is used. Yet, in the context of the present invention, it turned out to be advantageous, if the excitiation unit comprising the light sources and the detection unit comprising the detector entities are located in separate housings with the result that the improved real time PCR instrument comprises two separate optical units.

For the excitation unit, either monochromatic excitation or polychromatic excitation may be used according to the invention. For monochromatic excitation, either a monochromatic LED (light emitting diode) or a monochromatic laser may be used. It is also possible to use instruments with two or multiple LEDs or monochromatic lasers but only using one at a time in an individual measurement. For polychromatic excitation, a white LED, a Halogen lamp or a Xenon lamp may be used. In this case either a filter wheel or other means for moving appropriate filters into and out of the excitation beam need to be installed in the instrument such that light with different selective excitation wavelengths can be provided.

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The optical features of the excitation unit according to the invention are selected according to different parameters that have to be considered:

First, the shortest excitation wavelength should be longer than 360 nm and preferably longer than 400 nm, since short wavelength radiation has compartively low transmission rates through conventionally used optical material which is used for real time PCR reaction vessels such as glas or plastic. In addition, excitation with higher wavelengths for example in the range of 400-500 nm avoids interference of intrinsic fluorescence from the material of the reaction vessels which might disturb monitoring of the reaction.

In a prefered embodiment of the invention, the excitation unit consists of only one light source, because according to the present invention even with a single light source it is possible to perform a multiplex detection in real time PCR. Moreover, a multiplex detection in real time PCR is even possible with a single, monochromatic light source.

In one embodiment, the excitation unit comprises a blue LED emitting at 470 nm. Fig. 1 shows the excitation spectra for different fluorescent dyes which may be used according to the present invention. Among these dyes, Fluorescein as a frequently used FRET donor compound or TaqMan reporter dye known by a person skilled in the art can be excitated with a single light emitting diode having an excitation wavelength of 470 nm, whereas frequently used FRET acceptor dyes are only excited marginally with 470 nm LED.

In addition, a second UV-LED emitting in between 400 and 430 nm, preferably at 415 nm may be used optionally for exciting other FRET donor compounds having a shorter excitation maximum as compared to Fluorescein.

More precisely, the blue 470 nm LED is capable of exciting Fluorescein, which is used in the HybProbe format as donor dye in combination with 4 different acceptor (reporter) dyes. On the other hand, typical FRET acceptor dyes such as LC-Red-640 or Cy5 are only excited marginally by the 470 nm LED. The blue LED may also excite the reporter dyes FAM and HEX or VIC in dual color TaqMan assays. In addition, Fluorescein is used as reporter dye in the Single Label Probe (SLP) format.

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Moreover, the blue LED is used to excite SybrGreenI for non probe based detection of amplification products.

The optional UV LED may be used in the HybProbe format to excite a short wavelenth donor dye in combination with approriate reporter dyes that emit fluorescence at comparatively short wavelengths. Moreover, a third LED emitting at 580 nm may be included.

For the detection unit, different modes of detection which are known in the art can be used according to the invention. For example, it is well known in the art to use several photodiodes characterized in that each photodiode is capable of detecting fluorescent light of a specific wavelength. In one aspect of the invention, a detection system having exactly five or six channels using photodiodes as detectors is provided. As it will be shown, such embodiments already enable for a simultaneous 4 color FRET Hybridization Probe detection.

The optical features of the detection unit according to the invention are selected according to the following different parameters:

First of all, the longest emission wavelength that should be detected is around less than 800 nm, and preferably less than 730 nm, since detection of higher wavelength signals is affected by a possible background of putative infrared radiation by the heating part of the instrument. Moreover, long wavelength infrared dyes which are known in the art have a suboptimal quantum yield, are less stable and thus more difficult to couple to a desired hybridization probe.

As a result, the different detection channels should have central detection maximae between 500 and 800 nm and preferably between 520 and 730 nm. In this context, fig. 2 shows the emission spectra for different fluorescent dyes which may be used according to the present invention.

Within the range of 500 and 800 nm, the detection channnels can be defined exactly by means of seleting appropriate filters which the light beam has to pass prior to entering the respective photodiode. For appropriate selection, the following parameters have been identified by the inventors to be considered:

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First, the central wavelength detection maximum of each channel should be chosen dependent on the maxima of the emission spectra of those fluorescent dyes that shall be detected predominantly. As a rule of thumb, for appropriate detection sensitivity, the maximum of the fluorescent emission from the dye to be detected should not differ more than +/-10nm from the centrally detected wavelength of each channel.

Moreover, all detection channels should be able to detect discrete emissions, i. e. the spectral overlap should be minimized as far as possible. In this regard, it is advantageous, if the half band widths of each channel is 40 nm or less. More advantageously, the half band widths of each channel is 20 nm or less.

Preferably, crosstalk of the fluorophores to be detected in neighboring channnels is less than 70%, more preferably less than 50% und most prefered less than 30%. Another important criterium in this regard is the blocking value, which is indicative for the maximum intensity that is detected outside the specified transmission range of the channel (filter). Prefably, this value is less than 10^{-3}.

Based on these criteria, it has been proven to be advantageous, if the centrally detected wavelenghts of each of the 5-6 channels according to the invention are separated from each other by more than 25 nm and preferably even more than 30 nm.

In one embodiment, said centrally detected wavelengths are selected from a group of range of wavelengths, said group consisiting of 520-540 nm, 545-565 nm, 570-590 nm, 600-620 nm, 630-650 nm, 660-680 nm, and 700-720 nm.

Moreover, in one specific embodiment consisting of 6 detector channels said channels having centrally detected wavelengths at around 530, 580, 610, 640, 670, and 710 nm +/- 5 nm or 530, 555, 610, 640, 670, and 710 nm +/- 5 nm or preferably +/-2nm.

In another specific embodiment consisting of 5 detector channels said channels have centrally detected wavelengths at around 530, 610, 640, 670, and 710 nm +/-5nm or preferably +/-2nm.

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In a third specific embodiment consisting of 4 detector channels said channels have centrally detected wavelenghts at around 610, 640, 670, and 710 nm +/- 5 nm or preferably +/-2nm.

As an example, fig. 3 shows the 6 detection channels of a prefered embodiment and the emission spectra for different fluorescent dyes which may be used according to the present invention. In this embodiment, the six channels are separated by at least 25 nm spectral distance to minimize the crosstalk from each reporter dye into neighboring detection channels. The detection unit is capable of detecting SybrGreenI and the TaqMan reporter dye Fluorescein/FAM in the channel having its central wavelength detection maximum around 530 nm. In addition, the system is preferentially capable of detecting HEX/VIC in a second channel around 560 nm. Further long wavelength detection channels are capable of detecting HybProbe acceptor dyes and optionally long wavelength SLP reporter dyes.

The excitation and detection unit are connected by a multiple-leg fiber bundle. Emitted light from the reaction vessels (e.g. the glas capillaries) is homogeneously distributed using a lightpipe and is transmitted into six glas fiber bundles. These bundles of 50 µm single glas fibers transmit the light into each of the six detection channels.

This set up of excition unit and detection unit located in separate housings provides two advantages compared to the optical unit as disclosed in WO 97/46712: Homogeneous distribution of emitted light into all six detection channels and mechanical decoupling of the excitation and detection unit. This enables highly precise positioning of the excitation unit towards the reaction vessels to become monitored (e. g. capillary tips) without moving the detection unit. Moreover, the number of necessary dichrodic mirrors is minimized. An example of such a set up is shown in fig. 4, which discloses a possible embodiment of the invention. As it can be seen, excitation and detection unit are located in different housings.

In addition, the system according to the invention comprises a color compensation tool as disclosed in detail in US 6,197,520 which is incorporated into this application in its entirety by reference. The corresponding software enables for measuring fluorescence throughout a range of temperatures and correcting for temperature dependent spectral overlap of the 6 detector channels disclosed above.

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In principle this is achieved by

a) providing calibrator solutions for each fluorescent entity which is incorporated in the multiplex experiment to become performed

b) monitoring temperature dependent fluorescene of each of said solutions in each of the detector channels in order to determine the temperature dependent amount of crosstalk between different channels, and

c) generating a color compensation data set containing correction values.

B) **Test formats and selection of dyes**

In the context of the present invention, the term „multiplex PCR" is understood as a PCR reaction characterized in that 2 or more different amplification products are generated by means of using 2 or more pairs of amplication primers in the same PCR reaction.

Also in the context of the present invention, the term „multicolor real time PCR" is defined as an real time PCR assay characterized in that 1 or more different amplification products generated either in a multiplex PCR or in a monoplex PCR (using only 1 pair of amplification primers) are detected by differently labeled hybridization probes.

A major aspect of the present invention is based on usage of differently labeled hybridization reagents, each reagent comprising a pair of FRET hybridization probes comprising a pair of two fluorescent dyes which interact with each other on the principle of fluorescence resonance energy transfer (FRET). According to the invention, appropriate dye pairs can be selected for the labeling of a hybridization reagent which may be used for detection in at least three or four and preferably as much detection channels as possible.

More precisely, such a hybridization reagent is composed of two adjacently hybridizing oligonucleotides, appropriately labelled such that together they can act according to the FRET-HybProbe detection format as disclosed in WO 97/46707, WO 97/46712, and WO 97/46714). In many cases, it is sufficient if the

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hybridization reagent consists of a single oligonucleotide or in case of the FRET hybprobe format, of a pair of oligonucleotides acting together as a donor probe and an acceptor probe. Yet, in other cases there may exist many other sequence variants in the target sequences which need to be detected. Thus it may be impossible to detect the sequences of all members by just using just one pair of FRET oligonucleotide hybridization probes.

For those cases, a hybridization reagent may consist of 1, 2 or more hybridization probes, which are similar and bind to homologous sequences, but differ from each other by 1, 2, 3 or more mononucleotide-, dinucleotide- or trinucleotide exchanges, deletions or additions. In case of the hybridization reagent being a pair of FRET hybridization probes, said hybridization reagent may consist of 1, 2, 3, or more FRET donor oligonucleotide probes and/or 1, 2, 3, or more acceptor oligonucleotide probes. In this case all donor probes may be similar, but differ from each other by mononucleotide-, dinucleotide- or trinucleotide exchanges, deletions or additions. As well, all acceptor probes may be similar, differ from each other by mononucleotide-, dinucleotide- or trinucleotide exchanges, deletions or additions.

In addition it is also within the scope of the present invention, if multi-color FRET detection is performed wherein one member of said pair of labeled hybridization probes is replaced by an appropriately labeled primer as disclosed in Bernard, P. S., et al., Analytical Biochemistry 255 (1998) 101-107. Moreover, it is also within the scope of the present invention, if instead of an increase in fluorescenct emission from a respective FRET acceptor compound, the decrease in fluorescent emission of one, several, or all FRET donor moieties is monitored as being indicative for the presence of the target nucleic acid to be detected.

In addition to the various possibilites for performing multi-color detection based on the FRET principle, the system of the invention also allows for the performance of other detection formates such as detection of the target nucleic acid by means of SybrGreenI, multiplex detection using differentially labeled Molecular Beacons, Single Labeled Probes or dual color TaqMan detection. As it known in the art, dual color TaqMan assays may be based on the use of Cy 5 (Amersham) as Quencher compounds in combination with FAM as a first standard reporter dye and either HEX or alternatively VIC as a second reporter compound.

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In principle, there exist multiple possibilities of fluorescent dye combinations, which together may act as a FRET pair (Review: Resonance Energy Transfer Editors: Meer, B Wieb van der et al., VCH Publishers INC., 1994). However, based on the instrumental preconditions it is not trivial for person skilled in the art to develop a multi-color test which combines multiple FRET hybridization reagents that do not interfere with each other in such a way that sensitivity and/or specificity of a respective real time PCR experiment are affected.

Regarding the selection of approriate pairs of FRET hybridization probes for example, the dyes used for FRET donors and FRET acceptors can not be chosen arnbitrarily but need to fulfill at least the following requirements:

- sufficient solubility and capability of being coupled to an oligonkleotide
- stability of the dyes under PCR thermocycling conditions
- sufficient and reproducible emission intensity and quantum yield
- low temperature dependence of emission intensity
- essentially no spectral shifts under different chemical conditions
- essentially no spectral shifts when coupled to different oligonucleotide sequences
- discrete spectral emission maxima
- availability of a suitable partner dye for performing a FRET process

As a consequence, one important aspect of the system according to the invention is focussed on identification of multiple Fluorescence Resonance Energy Transfer (FRET) dye pairs usable in the HybProbe format for as many detection channels as possible. Excitation and emission spectra of dyes FRET dye pairs that enable a 4- or 5-plex multi-color detection set up are already shown in Fig. 1-3. Alternatives wil be be described below.

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In this regard, the following table summarizes various FRET acceptor dyes and TaqMan reporter dyes which have succesfully been used so far in the indicated detection channels performing either a 4-color FRET Hybridization Probe assay or a dual color TaqMen asssay:

Table 1

Channel	FRET Acceptor dye	TaqMan Reporter dye
530 (also detection of SybrGreenI)	-	Fluorescein (Fam) Coumarine
555	Rh6G Tamra	Hex Vic Tamra
580		Tamra ROX
610	LCRed610	TexasRed
640	LCRed640	
670	Cy5 Bodipi 655 Alexa 647	
710	Ja286 Cy5.5 LCRed705	

Yet, for several of the different reporter entities indicated above, different FRET donor moieties (Donor) and TaqMan quencher compounds (Q) respectively have to be used depending on the excitation wavelength which is available due to the presence of either one, two or three different LEDs. Therefore, different possibilities of appropriate FRET pairs or TaqMan combinations are summarized in the following more comprehensive table:

Table 2

	LED	530	555	580	610	640	670	710
FRET:								
Donor: Atto 425 WI 343	410	-	Rh6G TAMRA	-	LCRed 610	LCRed 640	Cy5 Bodipy655 Alexa647	JA286 LCRed705 Cy5.5
Donor: Fluores-cein	470	-	-	-	LCRed 610	LCRed 640	Cy5 Bodipy655 Alexa647	JA286 LCRed705 Cy5.5
Donor: LCRed 640	590	-	-	-	-	-	Cy5 Bodipy655 Alexa647	JA286 LC705 Cy5.5
TaqMan:								
Q: Arbi-trary	410	Cou ma-rine	-	-	-	-	-	-
Q: Tamra	470	Fam	Hex Vic	-	-	-	-	-
Q: Cy5	470	Fam	Hex Vic TAMRA	TAMRA Rox	-	-	-	-
Q: BHQ	470	Fam	Hex Vic TAMRA	TAMRA Rox	-	-	-	-
Q: Cy5	590	-	-	-	Texas-Red	-	-	-
Q: BHQ	590	-	-	-	Texas-Red	-	-	-

The dyes mentioned in the tables above are available as follows:

LC-Red-705 and LC Red-640 are available from Roche Applied Science (Cat. No. 2 015 161 and 2 157 594)

LC-Red 610 is synthesized according to standard protocols using a fluorescent dye as disclosed in US 5,750,409, compound II

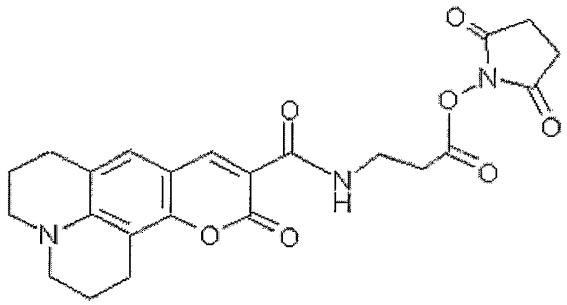
JA 286 is disclosed in EP 0 747 447, example 1. For this dye, it is advantageous, if this dye is connected to the hybridizing oligonucleotide by a long non hybridizing spacer moiety of five nucleotide residues.

Cy-5 NHS-ester is available from Amersham, (Cat. No. PA 15100) and Cyc 5.5 Phosphoramidite is also available from Amersham (Cat. No 271799 01).

Bodipy 650/665 NHS-Ester and Alexa 647 NHS Ester are available from Molecular Probes (Cat. Nos D1 000 1 and A 2 000 6).

Atto 425-NHS ester is available from Attotec (Cat. No. AD 425-3).

WI 343 is a derivative of Coumarin 343 (Aldrich, Cat. No. 55 804 654), which is coupled with a linker (beta alanin) and transformed to the succinimidyl ester according to standard methods:



Wi 343

In addition, all TaqMan dyes are available from different suppliers known by a person skilled in the art, for example Molecular probes.

The BlackHole Quencher (BHQ) is available from Biosearch Technologies (Cat. No. BHQ 1-3).

Appropriate labeling of oligonucleotide probes may be performed by conventional methods known by a person skilled in the art. In particular, labeling may be practiced using either fluorescent compounds that comprise an activated NHS-ester or fluorescent compounds linked to a Phosphoramidite, such that labeling can be

performed during a de-novo oligonucleotide synthesis itself. Furthermore, a Fluorescein-labeled probe may be prepared by using a Fluorescein Controlled Pore Glass particle as a solid support (Roche Applied Science Cat. No. 3 138 178) for oligonucleotide synthesis.

C) Methods and Kits

In a further aspect, the invention is directed to methods of performing multiplex PCR by means of using all different embodiments of the systems, instruments and compositions disclosed above.

In particular, the present invention is directed to a method for amplifying and detecting multiple target DNA sequences comprising

- providing a composition or reaction mixture according to the invention,
- subjecting said reaction mixture to a thermocyling protocol such that amplification of said multiple target sequences can take place, and
- monitoring hybridization of each of said pairs of FRET hybridization probes at least once after a plurality of amplification cycles.

In a specific embodiment, hybridization is monitored at least once in a temperature dependent manner.

The composition or reaction mixture predominantly comprises at least 3, preferably 4-5, and most preferably exactly 4 pairs of FRET hybridization probes, each pair of hybridization probes consisting of a FRET donor probe carrying a FRET donor moiety and a FRET acceptor probe carrying a FRET acceptor moiety having an emission maximum between 550 and 710 nm.

In addition, such a composition or reaction mixture according to the invention may comprise one or several or preferably all compounds and reagents selected from the following list:

- Buffer, applicable for a polymerase chain reaction

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- Deoxynucleoside triphosphates
- Template dependent DNA polymerase, preferably thermostable,
- at least one pair or several pairs of amplification primers

In yet another aspect, the invention is directed to a kit comprising at least a first set of amplification primers and at least three, four, or five pairs of FRET hybridization probes.

In addition, such a kit according to the invention may comprise one or several other compounds and reagents selected from the following list:

- Buffer, applicable for a polymerase chain reaction
- Deoxynucleoside triphosphates
- Template dependent DNA polymerase, preferably thermostable,
- At least one or multiple pairs of amplification primers

Such a kit may also comprise an internal control DNA, which can be amplified and detected using the same primers and probes as used for the detection of any target nucleic acid. Each of the components disclosed above may be stored in a single storage vessel. Yet, any combination of components for storage within the same vessel is possible as well.

D) **Best mode of the invention:**

The best mode of the invention known by the inventors at the date of filing the application is as follows:

In this prefered embodiment, a system according to the invention provides a photometer with one or two excitation sources and six detection channels. A blue (470 nm) and optionally an UV (410 nm) light-emitting diode are combined with

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detection channels having central detection wavelengths at 530, 555, 610, 640, 670 and 710 nm.

The photometer of the improved real time PCR instrument comprises two separate optical units. As disclosed in fig. 4, excitation and detection unit are located in different housings.

(I) An **excitation unit** with one or two LED's: A blue LED emitting at 470 nm and optionally a violet LED emitting at 415 nm. The blue LED is capable of exciting SybrGreenI and Fluorescein. Fluorescein is used in the HybProbe format as donor dye in combination with 4 different acceptor (reporter) dyes: Red 610, Red 640, Cy 5, Red 705. Furthermore the blue LED excites the reporter dyes FAM and HEX or VIC in dual color TaqMan assays. In addition, fluorescein is used as reporter dye in the Single Label Probe (SLP) format. The optional violet LED may used in the HybProbe format to excite a short wavelenth donor dye (Atto 425)) in combination with a reporter dye at 555 nm (Rh6G).

(II) A **detection unit** with six channels using photodiodes as detectors. The six channels are separated by at least 25 nm spectral distance to minimize the crosstalk from each reporter dye into neighboring detection channels. The detection unit is capable of detecting SybrGreenI and fluorescein/FAM in the 530 nm channel. In addition, the system is capable of detecting HEX/VIC and RH6G a in the 555 nm channel. The long wavelegth detection channels (610, 640, 670, 710 nm) are capable of detecting HybProbe acceptor dyes and optionally for long wavelength SLP reporter dyes.

The excitation and detection unit are connected by a six-leg fiber bundle. Emitted light from the glas capillaries is homogeneously distributed using a lightpipe and is transmitted into six glas fiber bundles. These bundles of 50 µm single glas fibers transmit the light into each of the six detection channels. This setup provides two advantages compared to the LC 1.2 optical unit: Homogeneous distribution of emitted light into all six detection channels and mechanical decoupling of the excitation and detection unit. This enables highly precise positioning of the excitation unit towards the capillary tips without moving the detection unit. An effective color compensation function allows simultaneous detection of multiple targets in different channels.

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Combined with these hardware and software features, multicolor capabilities of the system in order to perform Multiplex FRET Hybridization Probedetection have been established. In addition, the system of according to the invention allows for other non FRET-Hybprobe detection formats.

Based on the Hybridization Probe detection format, the system enables at least 4-color multiplexing of real-time quantification and melting curve analysis. Taking a melting curve resolution of six melting peaks per channel into consideration, up to 24 different PCR products are discernable using the photometer according to the invention. To enable this, new previously unknown FRET-acceptor dyes have been developed for detection channels with central emission wavelengths at 610 and 670 nm. In addition, the optional 410 nm LED provides an option for using a short wavelength donor dye in combination with an acceptor dye emitting at 555 nm thus providing a fifth HybProbe detection channel. Excitation and emission spectra of specifically useful dyes are shown in Fig. 1 and 2.

Using fluorescein as FRET donor dye two new FRET acceptor dyes could be identified being detectable in the 610 nm channel (Red 610) and in the 670 nm channel (Cy 5) respectively. Both dyes show high signal dynamics when used in the HybProbe FRET format. In addition, the spectral properties of the dyes fit perfectly into the photometer disclosed above thus minimizing the crosstalk into heterologous detection channels. Using a flexible multiple-channel color compensation algorithm it is shown that specific detection of all 4 FRET acceptor dyes (including the already existing dyes LC Red 640 and LC Red 705) from a single reaction is feasible over a temperature range from 40°C to 95°C.

When trying to identify a fifth FRET acceptor dye for the 555 nm channel it turned out that good FRET signal dynamics is inhibited by two effects:

(I) The fluorescein donor dye shows a high crosstalk into the 555 nm channel thus masking increasing signals of a potential FRET acceptor dye during the PCR process.

(II) A FRET acceptor dye emitting at 555 nm is itself directly excited by the 470 nm LED thus increasing the background fluorescence significantly and consequently lowering the detection sensitivity.

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A solution to these drawbacks was found by using a 410 nm UV LED for excitation of a short wavelength donor dye (Atto 425) in combination with a suitable 555 nm acceptor dye (Rh6G). In this combination, crosstalk from the donor into the 555 nm detection channel and direct excitation of the FRET acceptor were minimized and sufficient signal dynamics was achieved after color compensation.

Furthermore, it turned out that the short wavelength donor Atto 425 can even be used in combination with all long wavelength acceptor dyes. Unexpectedly, FRET signal dynamics of long wavelength acceptors in combination with Atto 425 are comparable to signal dynamics when used in combination with fluorescein. This observation provides an option to extend multi color features to a 5-channel format using just one donor dye.

Using the TaqMan detection format, dual color applications based on standard TaqMan reporter dyes FAM (detected at 530 nm) and HEX or VIC (detected at 560 nm) can be performed without major modifications of the TaqMan chemistry known in the art. The obtained detection sensitivity is comparable to the COBAS TaqMan instrument (Roche Molecular Systems).

Furthermore, the Single Labeled Probes (SLP's) format for melting curve analysis (WO 02/14555) is applicable within a system according to the invention. This format is based on quenching or, respectively, de-quenching of the probe-label after hybridization to its target sequence. The format requires just one probe terminally labeled with a single dye. Compared to HybProbe or TaqMan formats, SLP's thus enable a significantly less expensive setup of assays for integrated analysis of Single Nucleotide Polymorphisms (SNP's). By reporting melting temperatures, different alleles of an SLP can unambigiously be distiguished in a single reaction. Multiplexing options are provided by using different reporter dyes.

Possibilities for Real time multicolor PCR detection in the above disclosed instrument are summarized in the following table:

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Table 3

Assay Format	Detection Channels (Reporter Dyes)
SYBR Green I	530 nm (SYBR Green I)
HybProbes	610 nm (LC RED 610) 640 nm (LC RED 640) 670 nm (LC RED 670) 710 nm (LC RED 705)
Hydrolysis Probes (TaqMan Probes)	530 nm (FAM) 555 nm (VIC, HEX)
SimpleProbes	530 nm (Fluorescein)

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 Excitation specra of FRET donor and acceptor dyes for the present invention

Figure 2 Emission spectra of FRET donor and acceptor dyes for the present invention

Figure 3 Emission spectra of FRET donor and acceptor dyes and detection channels for the present invention

Figure 4 Photometer setup

Figure 5 4 color real time PCR quantification experiment of example 1

Figure 6 Primer/probe design of example 2

Figure 7 4-color Real time PCR melting curve experiment of example 2

Figure 8 Dual color TaqMan Real time PCR quantification experiment of example 3

Example 1

Quantitative real time PCR of Factor V DNA using different pairs of FRET hybridization probes labeled with different fluorescent compounds

For amplification of a Factor V DNA fragment, 4 different 100µl real time PCR reaction mixtures were set up as follows:

10^6 copies of a plasmid containing Factor V gene

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3 mM $MgCl_2$

500 nM each primers

200 nM FRET 3'Hybridization Probeaccording to Seq. A

200 nM FRET 5'Hybridization Probeaccording to Seq. B1, B2, B3 or B4, respectively.

In addition, PCR components from the LightCycler DNA Hyb Probes Kit (Roche Applied Science, Cat. No. 2158825) were used.

Primers and probes were used as follows:

Primer FactorV, forward:

5'-GAG AGA CAT CGC CTC TGG GCT A-3' (22-mer) (Seq. ID. No:1)

Primer FactorV, reverse:

5'-TGT TAT CAC ACT GGT GCT AA-3' (20-mer) (Seq. ID. No: 2)

A: 3'Fluorescein labeled Hybridization Probe

5'-AAT ACC TGT ATT CCT CGC CTG TC-3' (23-mer) (SEq. Id. No: 3)

B1: 5' Red610 Hybridization Probe

5'-AGG GAT CTG CTC TTA CAG ATT AGA AGT AGT CCT ATT-3' (36-mer)

B2: 5' Red640 Hybridization Probe

5'-AGG GAT CTG CTC TTA CAG ATT AGA AGT AGT CCT ATT-3' (36-mer)

B3: 5' Cy5 Hybridization Probe

5'-AGG GAT CTG CTC TTA CAG ATT AGA AGT AGT CCT ATT-3' (36-mer)

B4: 5' Red705 Hybridization Probe

5'-AGG GAT CTG CTC TTA CAG ATT AGA AGT AGT CCT ATT-3' (36-mer)

(B1-B4: Seq.Id. NO: 4)

Amplification was performed in a LightCycler instrument disclosed above as best mode of the invention according to the following thermocycling protocol:

	T[°C]	t[sec]	Ramp-rate[°C/sec]	Acquisition	Cycles
Denaturation	95	30	20.0	none	1
Amplification	95	10	20.0	none	
	55	20	20.0	single	45
	72	20	20.0	none	

Real time monitoring was performed with and without a color compensation algorithm and using the 2nd derivative threshold method over 45 cycles by measuring the fluorescence signals in a detection channel and using arithmetic background correction for normalization of initial fluorescence background intensities.

Results are shown in fig. 5a-5d. As can be seen in the figures, specific and quantitative amplification signals of all used FRET acceptor dyes could be obtained after color compensation in the corresponding 610, 640, 670 and 710 nm detection channels. Moreover, it is shown that the spectral overlap of the different dyes in the neighboring channels did not affect specific and quantitatve signalling when appropriate color compensation is performed.

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Example 2

4-color melting curve analysis

A 479 bp fragment of a specific plasmid containig a fragment of the human NAT-2 gene encoding N-Acetyl-Transsferase isozyme was amplified with specific primers and detected by fluorescence, using 4 different specific pairs of FRET Hybridization Probes. Whereas three probes were labeled at the 3'-end with Fluorescein, four detection probes were labeled 5' either with Light- Cycler-Red 610, 640, 705 or Cy5 and modified at the 3'-end by phosphorylation. A schematic drawing of this experimental set up is shown in fig. 6. As it can be deduced from the figure, there were four differently labeled FRET acceptor probes, but only three detection sites. This resulted in a competition of annealing of two detection probes.

Assay conditions were basically the same as disclosed in example 1 with the alteration that 3x106 copies of target DNA were used.

Primer forward:

5'-TGC CTT GCA TTT TCT GCT T-3' (19mer) (Seq. Id. No: 5)

Primer reverse:

5'-GAG TTG GGT GAT ACA TAC A-3' (19mer) (Seq. Id. No: 6)

3'-Fluorescein HybProbe 1:

5'-GAA ATT CTT TGT TTG TAA TAT ACT GCT CTC TC-Fluos-3' (32mer) (Seq. Id. No:7)

5'-Red610 HybProbe 1a:

5'-Red610-TGA TTT GGT CCA CGT ACC-3' (18mer) (Seq. Id. No:8)

5'-Red640 HybProbe 1b:

5'-Red640-TGA TTT GGT CCA AGT ACC C-3' (19mer) (Seq. Id. No:9)

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3'-Fluorescein HybProbe 2:

5'-GTT GGA GAC GTC TGC AGG TAT GTA TTC ATA GAC TCA A-Fluos-3' (37mer)
(Seq. Id. No:10)

5'-Red705 HybProbe 2:

5'-Red705-ATC TTC AAT TGT TCG AGG TT-3' (20mer)
(Seq. Id. No:11)

3'-Fluorescein HybProbe 3:

5'-ATT TCC TTG GGG AGA AAT CTC GTG CCC A-Fluos-3' (28mer)
(Seq. Id. No:12)

5'-RedCy5 HybProbe 3:

5'-Cy5-ACC TGG TGA TGA ATC CCT TAC TAT TTA GAA TAA GGA AC-3' (37mer)
(Seq. Id. No:13)

Amplification and subsequent melting curve analysis was performed in a LightCycler instrument disclosed above as best mode of the invention according to the following thermocycling protocol:

	T[°C]	t[sec]	Ramp-rate[°C/sec]	Acquisition	Cycles
Denaturation	95	600	20.0	none	1
Amplification	95	10	20.0	none	
	55	10	20.0	single	45
	72	20	3.0	none	
Melting Curve	95	60	20.0	none	
	40	60	20.0	none	1

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	80	0	0.1	cont	
Cooling	40	30	20.0	none	1

Results are shown in fig. 7. As it can be deduced from the figure, 4 color melting curve analysis using Hybridization Probes with the 4 different acceptor dyes could be performed using an approriate color compensation method. Hybridization Probe specific melting peaks could then be detected without any substantial crosstalk effects.

Example 3

Dual Color TaqMan Detection

A LightCycler instrument according to the best mode of the invention provided all means for sensitive detection of dual color TaqMan assays. The established TaqMan dye FAM and HEXs were well excited with the 470 nm LED and after using the instrument's color compensation function could differentially be identified in the 530 nm and 560 nm detection channels with high sensitivity. Transfer of dual color TaqMan assay protocols to the instrument according to the invention was simply performed by supplementing established assay conditions with BSA.

Different examples of successful dual color amplification and detection are shown in fig. 8. Fig. 8a shows that in mono-color experiments using either FAM or HEX, a detection sensitivity of about 1copy/µl for dual color TaqMan assays was obtained. (Only negative controls did not result in a significant increase in fluorescence after the PCR reaction). Fig,. 8b shows a dual color experiment, wherein a Hex labeled TaqMan probe was always used to detect 500 copies of a first target DNA and in the same reaction a FAM labeled TaqMan probe was used to detect different amounts (10^0-10^6 copies) of a second target DNA.

The dynamic range (being indicative for the relative difference in concentrations of the two targets to become detected) was about 10^3.

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US 6,197,520

US 6,369,893

WO 02/14555

WO 97/46707

WO 97/46712

WO 98/46714

Patent Claims

1. A composition or reaction mixture for performing multi-color real time PCR comprising at least 3, preferably 4-5 most preferably exactly 4 pairs of FRET hybridization probes, each pair of hybridization probes consisting of a FRET donor probe carrying a FRET donor moiety and a FRET acceptor probe carrying a FRET acceptor moiety having an emission maximum between 550 and 710 nm.

2. A composition or reaction mixture according to claim 1, wherein at least 3, preferably at least 4 and most preferably exactly 4 FRET donor moieties are identical.

3. A composition or reaction mixture according to claim 1, wherein all FRET donor moieties are identical.

4. A composition or reaction mixture according to claim 1, wherein at least 3, preferably at least 4 and most preferably exactly 4 FRET donor moieties are Fluorescein.

5. A composition or reaction mixture according to claim 1, wherein all FRET donor moieties are Fluorescein.

6. A composition or reaction mixture according to claims 1-5, wherein at least one additional FRET donor moiety is selected from a group consisiting of Atto425 and WI343.

7. A composition or reaction mixture according to claims 1-6, wherein one FRET acceptor moiety is selected from a group consisting of LC-Red 705, Cy5.5, and JA286.

8. A composition or reaction mixture according to claims 1-7, wherein at least one, two or three FRET acceptor moieties are selected from a group consisting of Cy5, LC-Red 640, and LC-Red 610

9. A composition or reaction mixture according to claims 1-8,

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wherein one FRET acceptor moiety is selected from a group consisting of Rh6G and TAMRA.

10. A system for performing multi-color real time PCR, comprising

- a real time PCR instrument, and
- a composition or reaction mixture according to claims 1-9.

11. A system according to claim 10, characterized in that said real time PCR instrument comprises

- at least 1 light source, preferably an LED
- at least 4 and preferably 5-6 fluorescent detector entities, each of said entities having central detection wavelengths which are distinct from each other by at least 25 and preferably at least 30 nm

characterized in that said detector entities are capable of

- simultaneously detecting maximum fluorescene emission of at least 3, preferably 4 and most preferably 5 differently labeled FRET Hybridization Probe pairs,
- simultaneously detecting maximum fluorescence emission of at least 2 differently labeled TaqMan hybridization probes, and
- detecting maximum fluorescence emission of SybrGreenI

- means for heating and cooling
- multiple reaction vessels for containing a reaction mixture.

12. A system according to claim 11, characterized in that said real time PCR instrument comprises exactly one light source.

13. A method for amplifying and detecting multiple target DNA sequences comprising

a) providing a composition or reaction mixture according to claims 1-9,

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b) subjecting said reaction mixture to a thermocycling protocol such that amplification of said multiple target sequences can take place,

c) monitoring hybridization of each of said pairs of FRET hybridization probes at least once after a plurality of amplification cycles.

14. A method according to claim 13, wherein hybridization is monitored at least once in a temperature dependent manner.

15. A real time PCR instrument comprising

- at least 1 light source, preferably an LED
- 5-6 fluorescent detector entities, each of said entities having central detection wavelengths which are distinct from each other by at least 25 and preferably at least 30 nm,

characterized in that said detector entities are capable of

- simultaneously detecting maximum fluorescene emission of at least 3, preferably 4 and most preferably 5 differently labeled FRET Hybridization Probe pairs,
- simultaneously detecting maximum fluorescence emission of at least 2 differently labeled TaqMan hybridization probes, and
- detecting maximum fluorescence emission of SybrGreenI

- means for heating and cooling
- multiple reaction vessels for containing a reaction mixture.

16. A real time PCR instrument according to claim 15 comprising exactly one light source.

17. An instrument according to claim 15-16, characterized in that said central detection wavelengths are selected from a group of range of wavelengths, said group conisiting of 520-540 nm, 545-565 nm, 570-590 nm, 600-620 nm, 630-650 nm, 660-680 nm, and 700-720 nm.

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Fig. 1

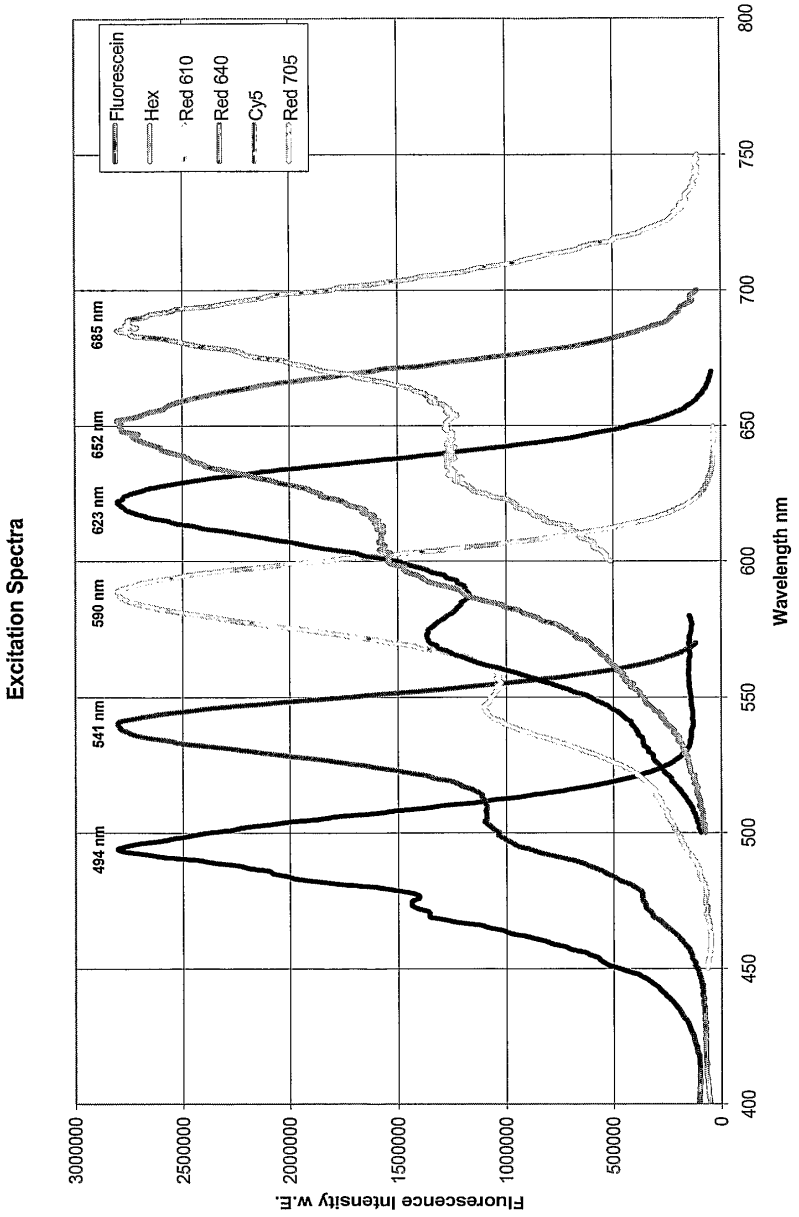
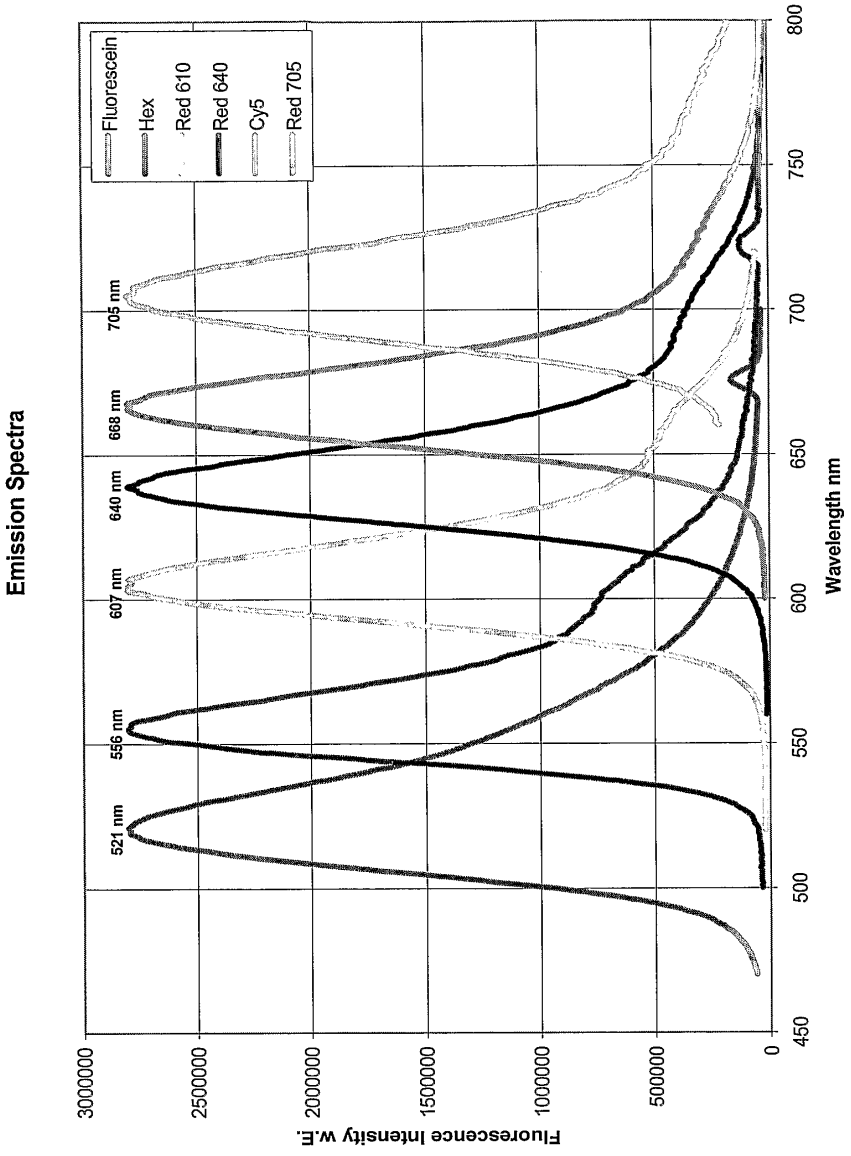

Excitation Spectra
Fluorescein
Hex
Red 610
Red 640
Cy5
Red 705
494 nm
541 nm
590 nm
623 nm
652 nm
685 nm
Fluorescence Intensity w.E.
3000000
2500000
2000000
1500000
1000000
500000
0
400
450
500
550
600
650
700
750
800
Wavelength nm

Fig. 2



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Fig. 3

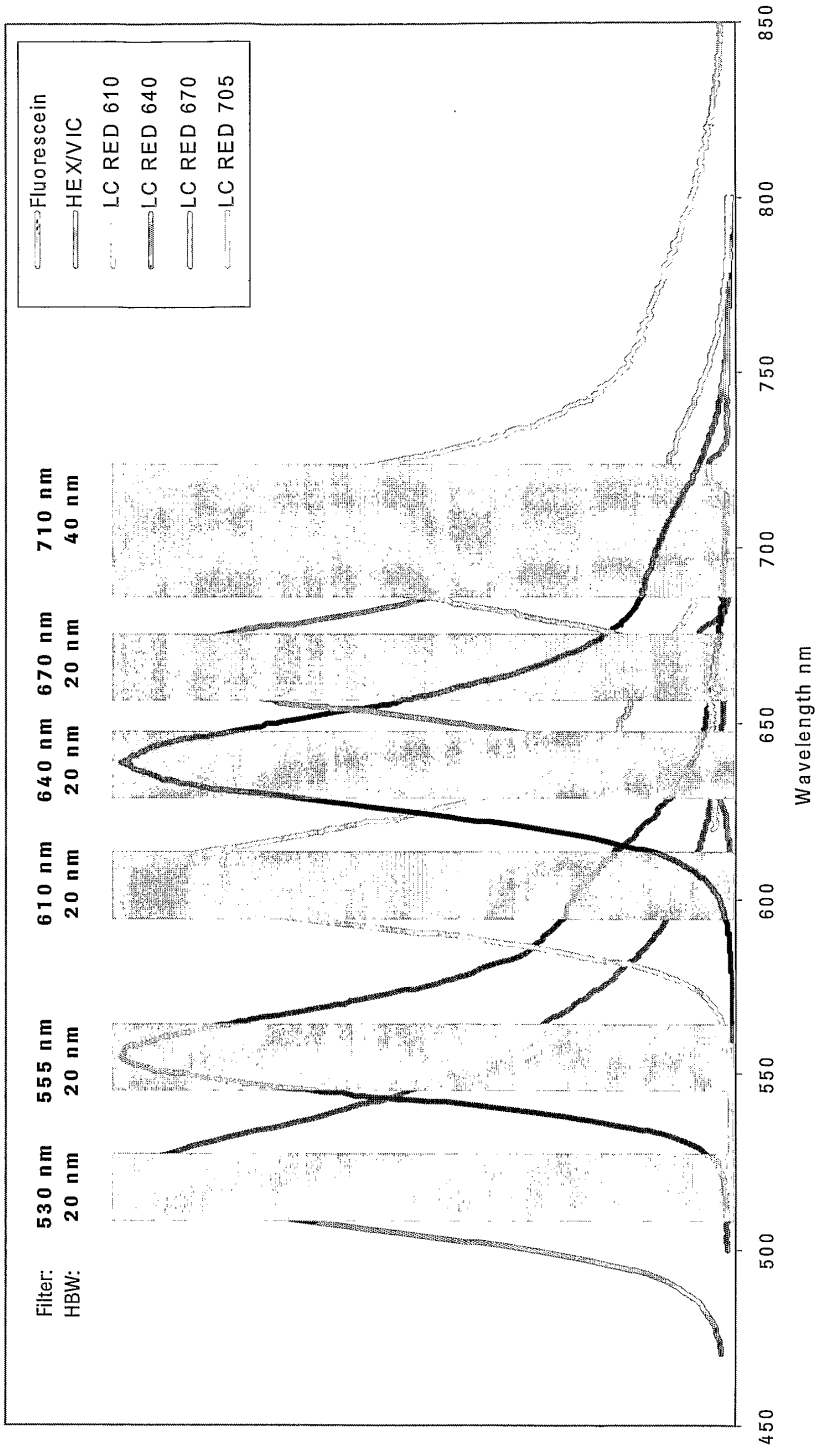
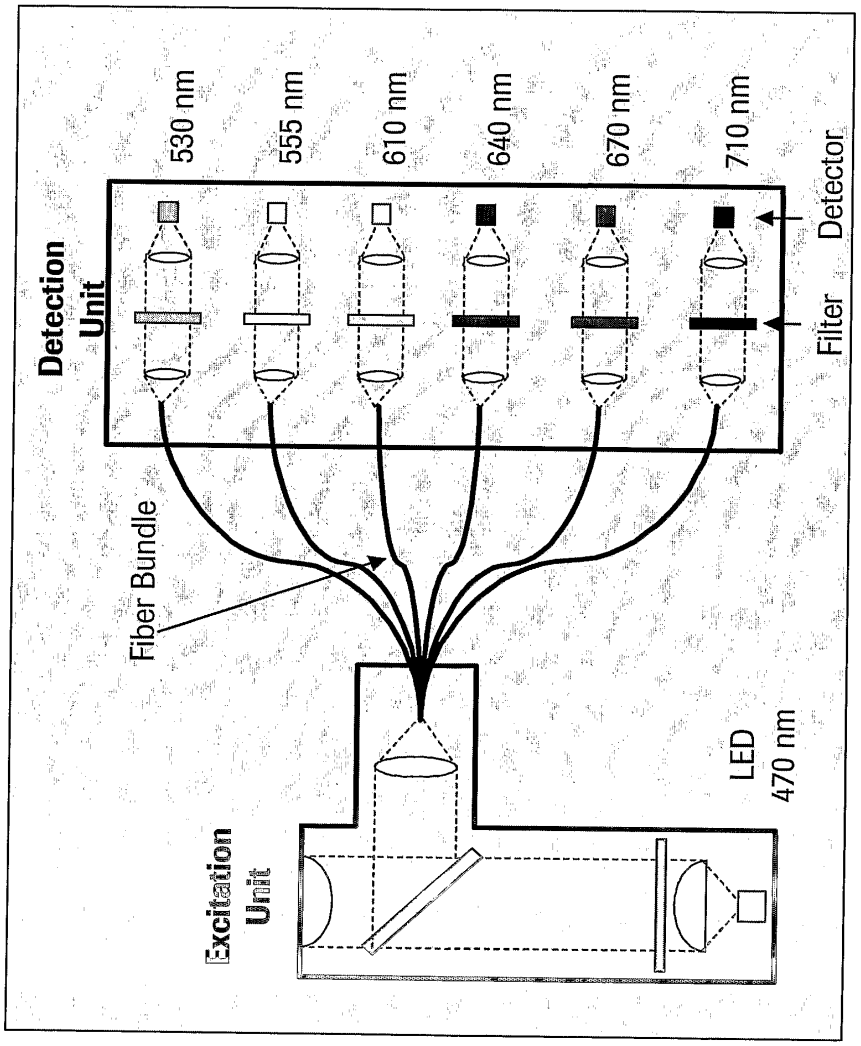

Fluorescein
HEX/VIC
LC RED 610
LC RED 640
LC RED 670
LC RED 705
Filter: 530 nm 555 nm 610 nm 640 nm 670 nm 710 nm
HBW: 20 nm 20 nm 20 nm 20 nm 20 nm 40 nm
450
500
550
600
650
700
750
800
850
Wavelength nm

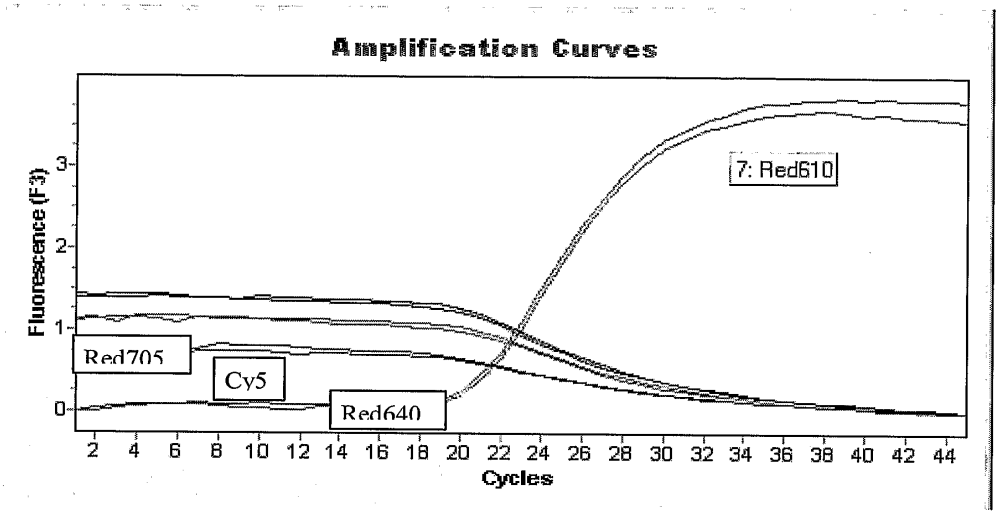
Fig. 4



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Fig. 5a

F3 = 610 nm
Without Color Compensation



With Color Compensation

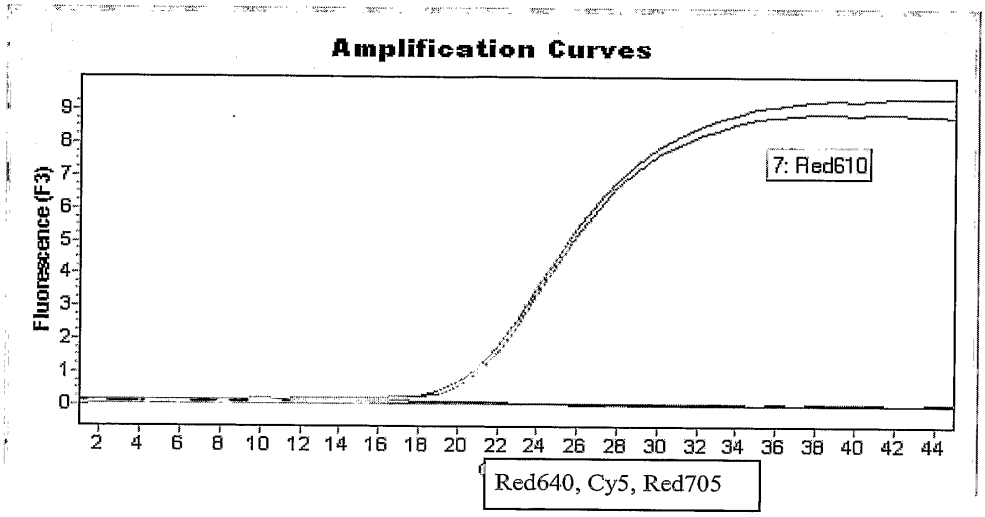
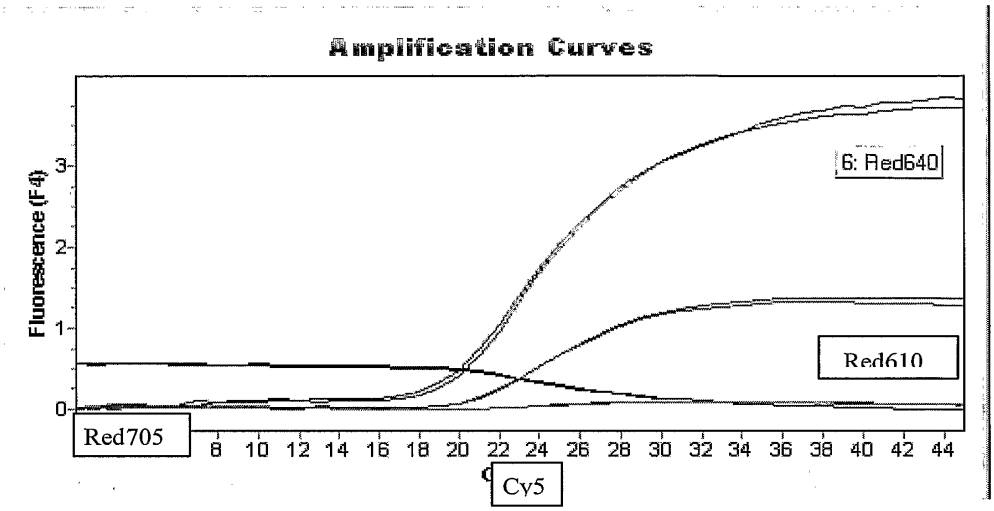


Fig. 5b

F4 = 640 nm
Without Color Compensation



With Color Compensation

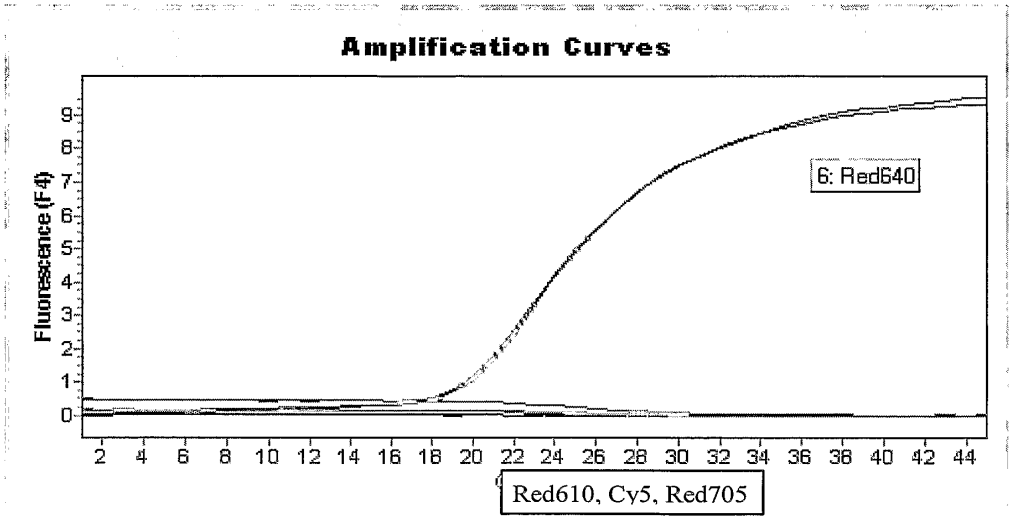
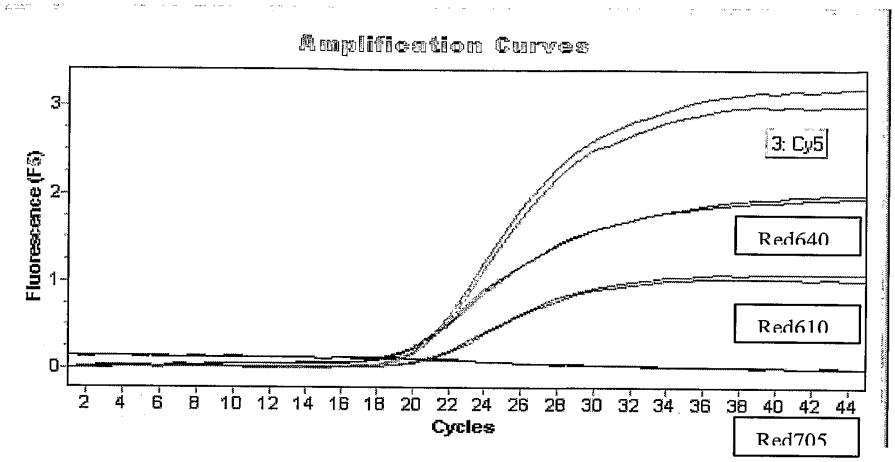


Fig. 5c

F5 = 670 nm
Without Color Compensation



With Color Compensation

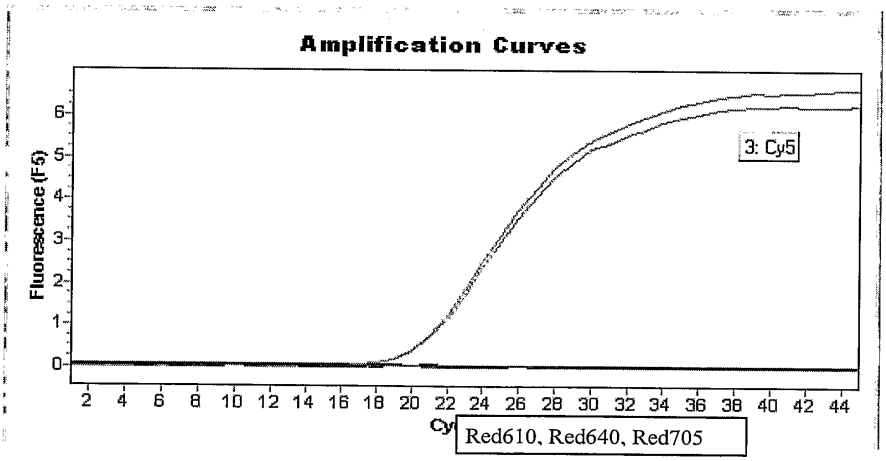
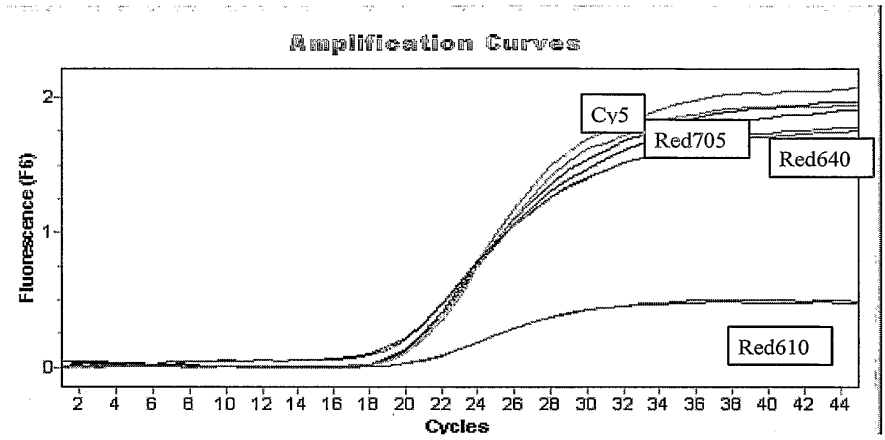
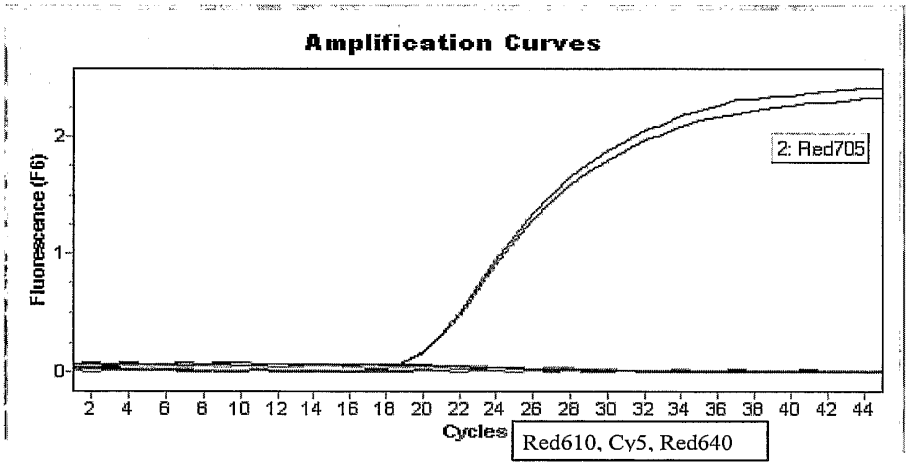


Fig. 5d

F6 = 710 nm
Without Color Compensation



With Color Compensation



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Fig. 6

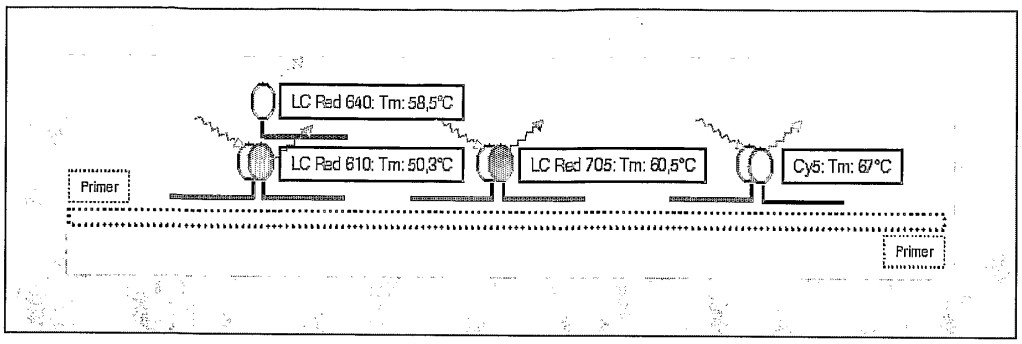
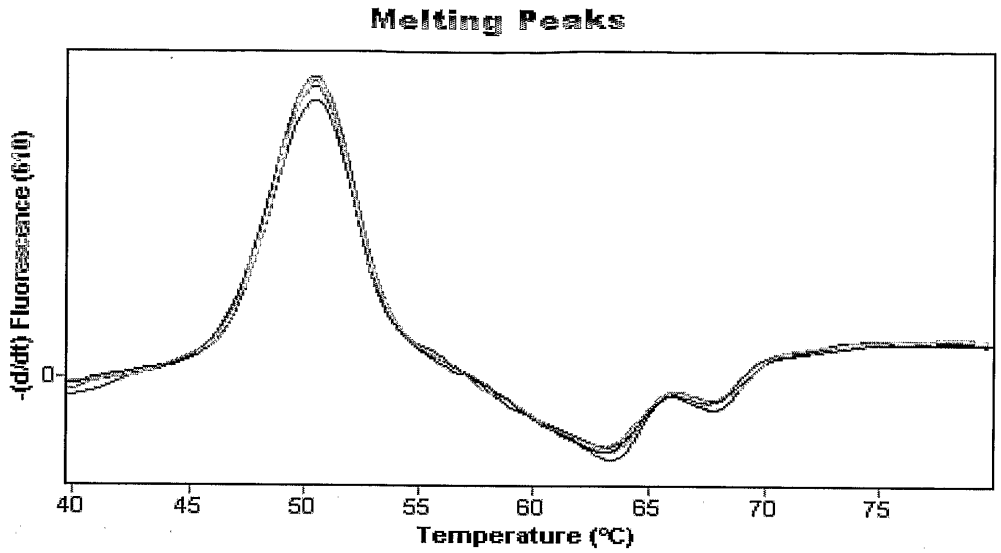
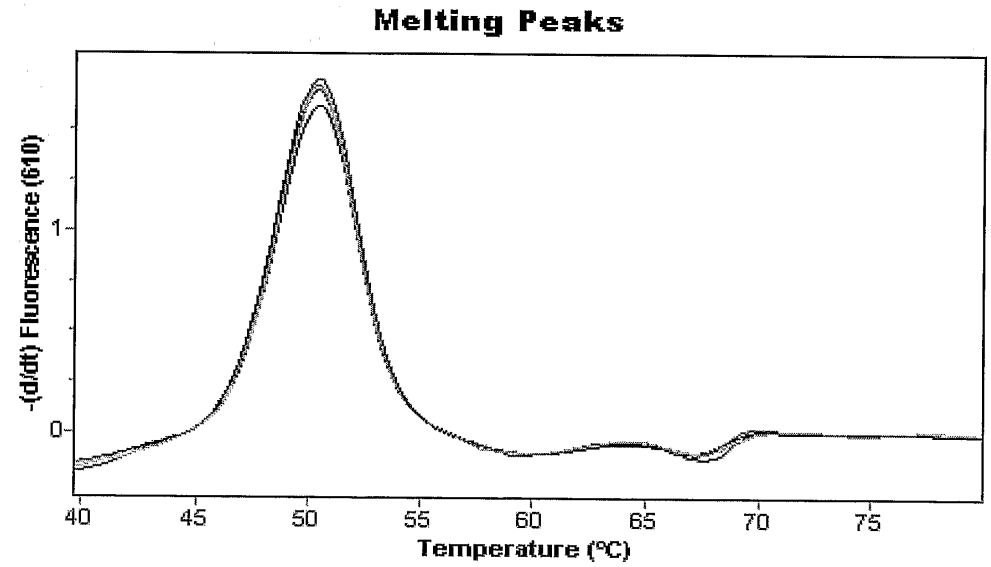

LC Red 640: Tm: 58,5°C
LC Red 610: Tm: 50,3°C
LC Red 705: Tm: 60,5°C
Cy5: Tm: 67°C
Primer
Primer

Fig. 7a

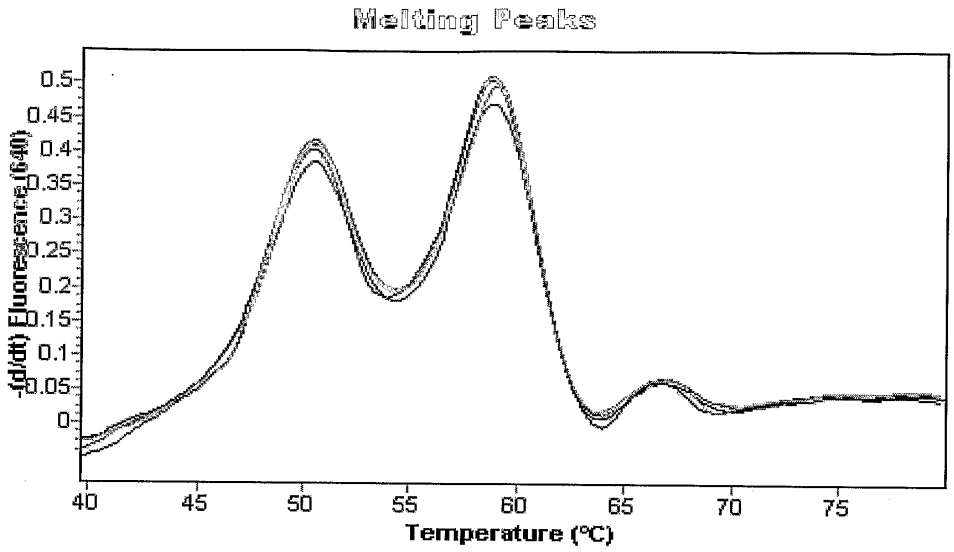


610 nm channel without color compensation

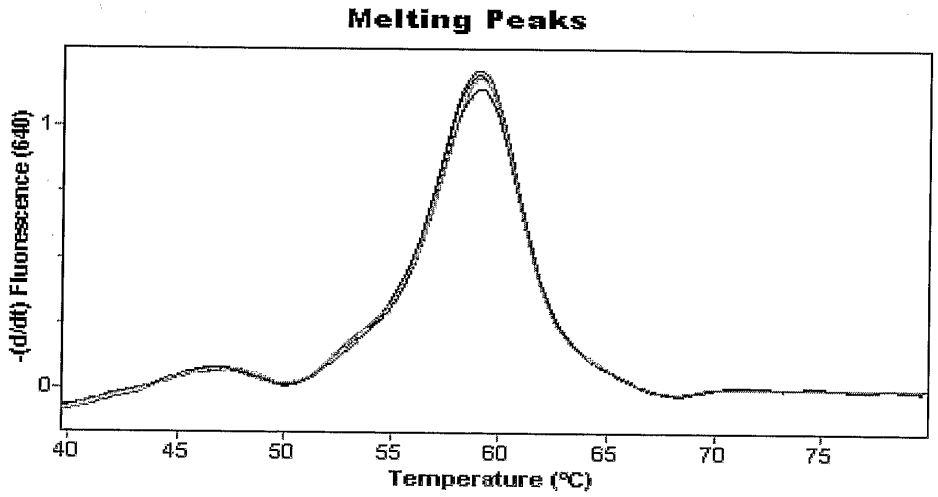


610 nm channel with color compensation

Fig. 7b

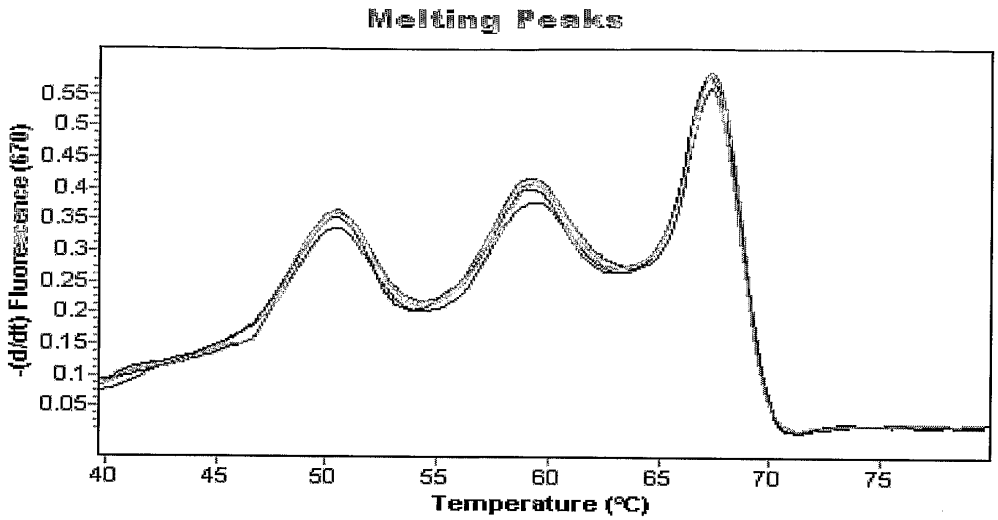


640 nm channel without color compensation

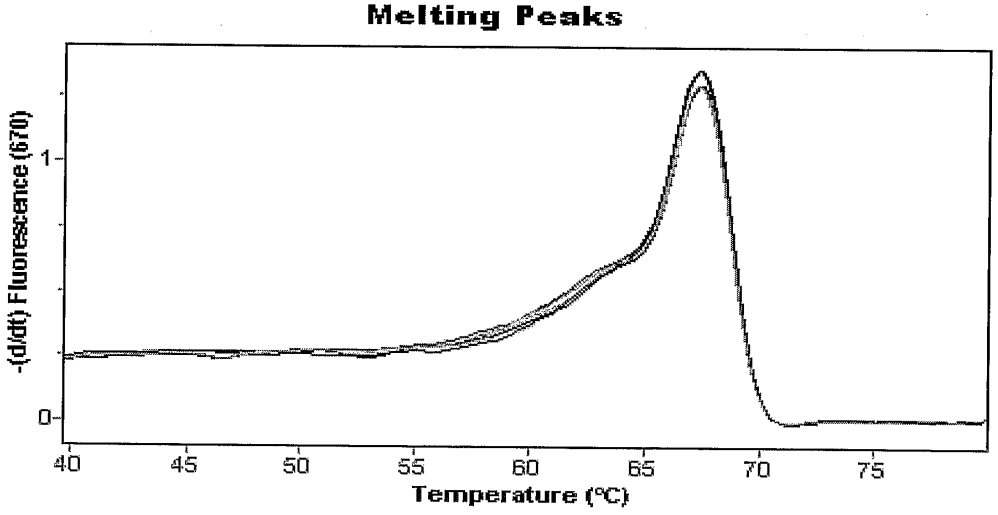


640 nm channel with color compensation

Fig. 7c

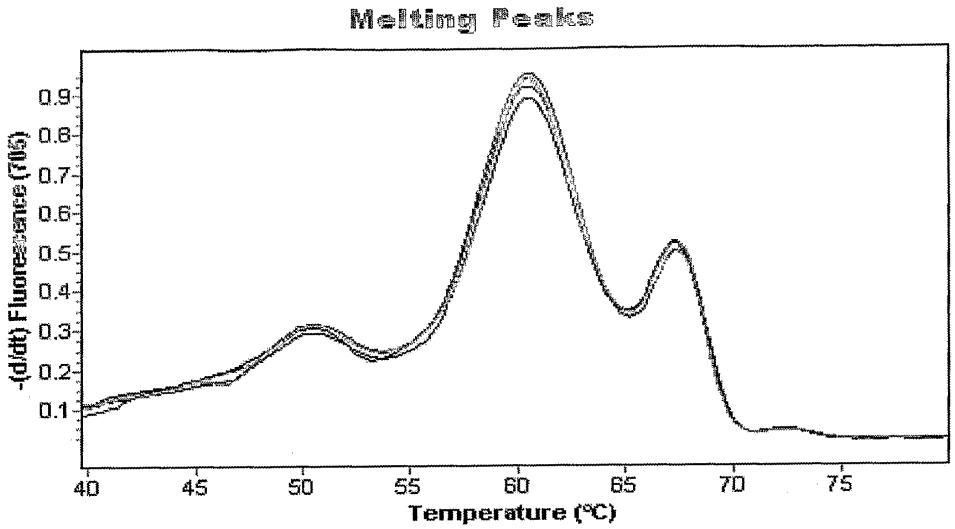


670 nm channel without color compensation

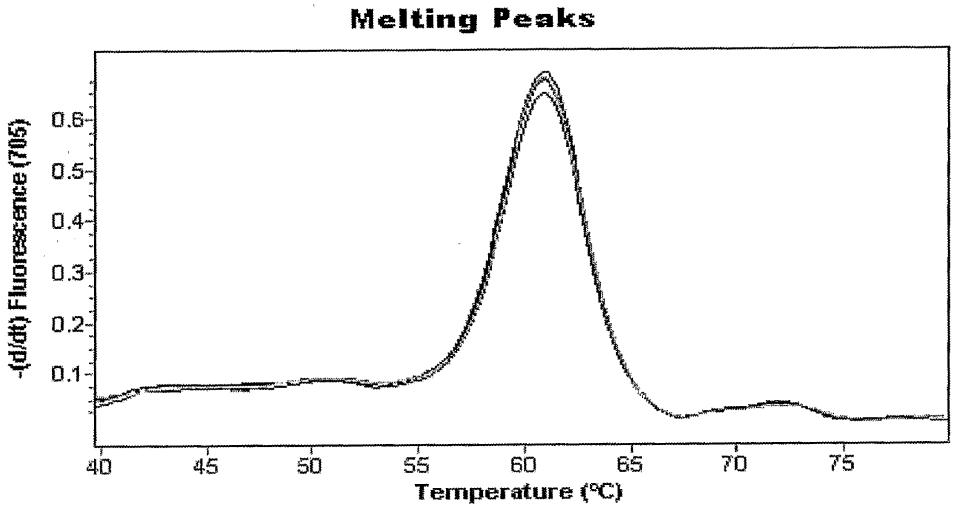


670 nm channel with color compensation

Fig. 7d



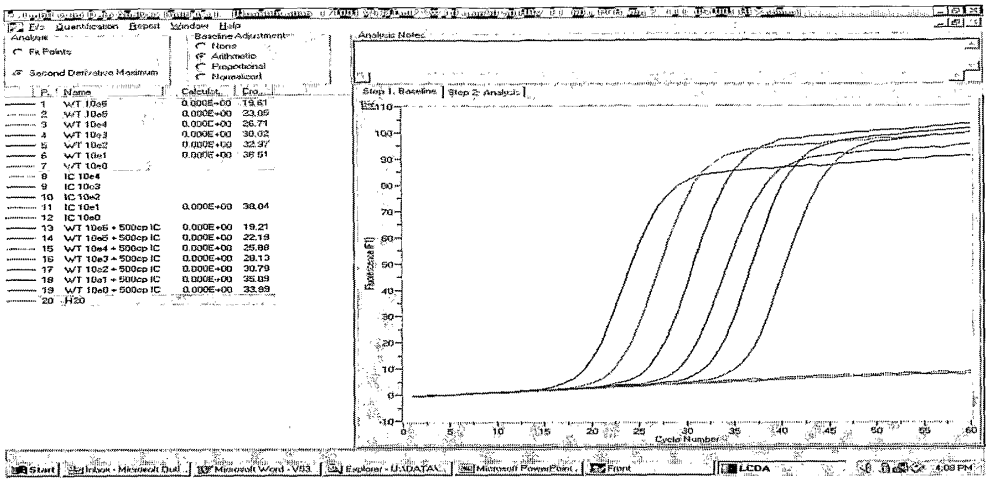
705 nm channel without color compensation



705 nm channel with color compensation

Fig. 8a

F1: 530nm (FAM)



F2: 560 nm (Hex)

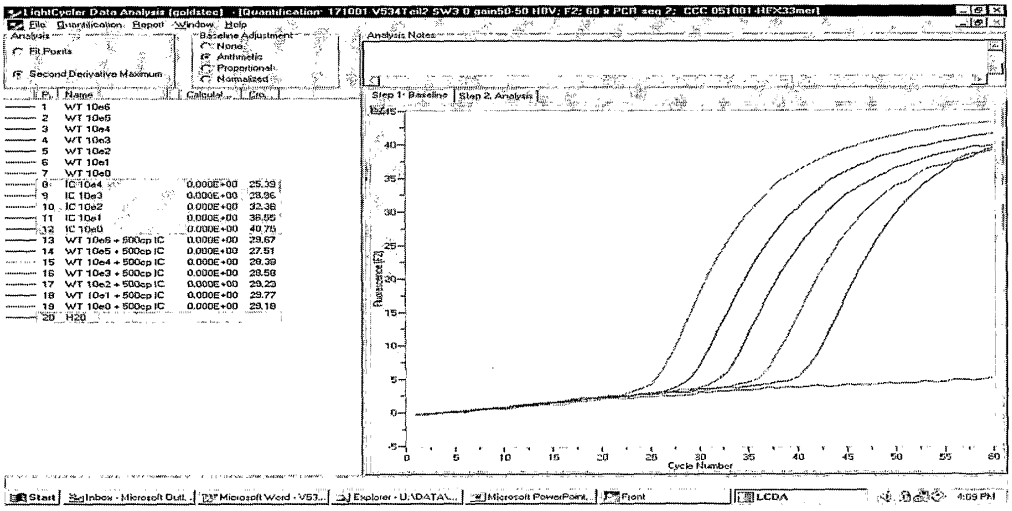
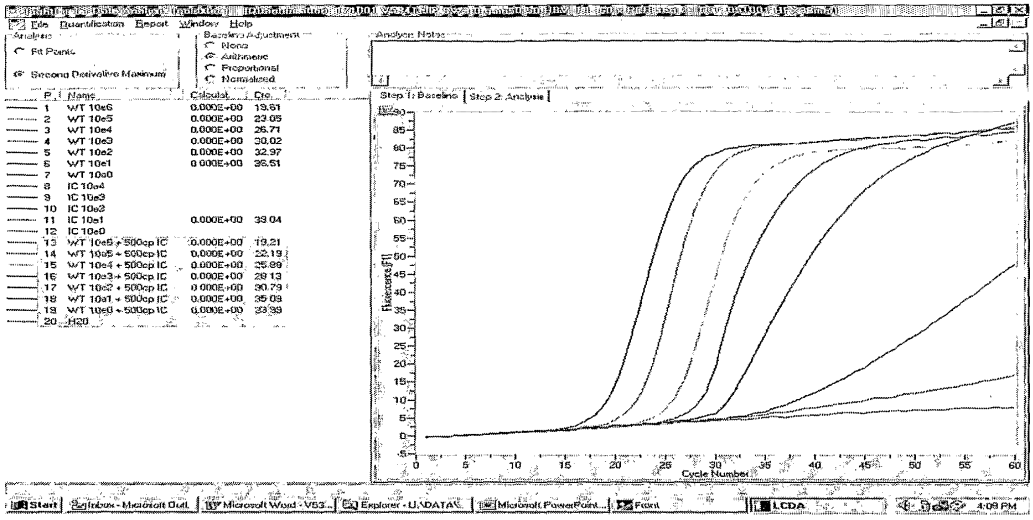
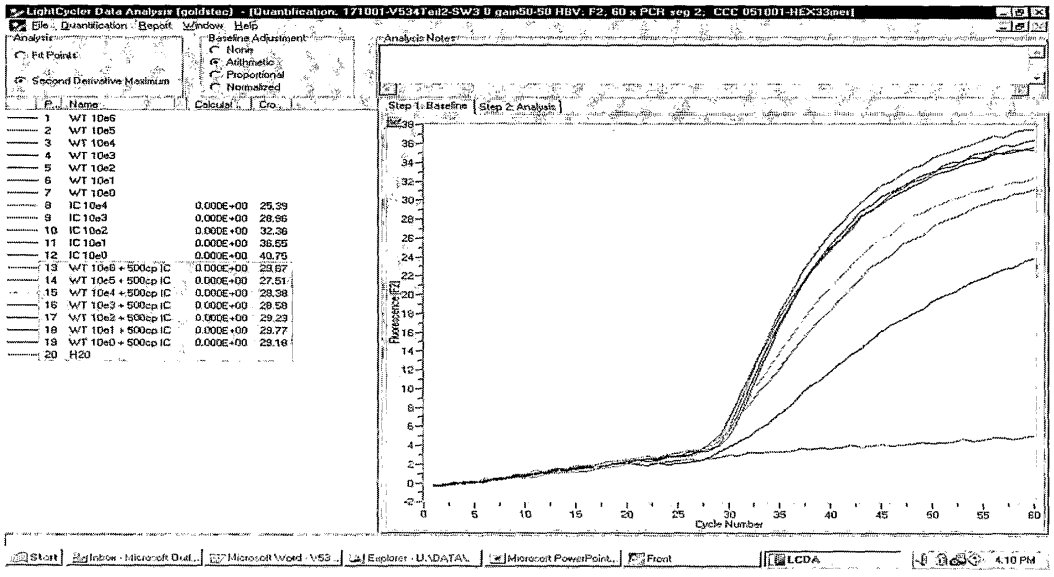


Fig. 8b

F1: 530nm (FAM)



F2: 560 nm (HEX)



SEQUENCE LISTING

<110> Roche Diagnostics GmbH

F. Hoffmann-La Roche AG

<120> Improved system for multi color real time PCR

<130> 21810 EP

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<151> 2003-04-04

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(54) **HOMOGENEOUS MULTIPLEX SCREENING ASSAYS AND KITS**

HOMOGENE MULTIPLEX-SCREENING-TESTS UND KITS

DOSAGES BIOLOGIQUES MULTIPLEXES HOMOGENES ET KITS ASSOCIES

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WO-A-03/100101 **WO-A1-03/020967**
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Description

TECHNICAL FIELD

[0001] This invention relates to homogenous *in vitro* multiplexed assays, including particularly homogeneous amplification assays, for screening biological samples for the presence of any of a large number of nucleic acid sequences.

BACKGROUND

[0002] A variety of assay techniques are available for testing a biological sample obtained from any of a variety of sources for the presence of a nucleic acid sequence that may indicate the presence, for example, of a particular bacterium, virus or other pathogen, including a particular strain or mutant. Assays are also available for testing such samples for the presence of a nucleic acid sequence of the subject's own genomic DNA that may indicate the presence, for example, of one or another disease-related genetic mutation. Assays may include oligonucleotide probes bearing detectable labels, for example, P^{32} or fluorophores. Nucleic acids, either DNA or RNA, in a sample may be probed directly. Alternatively, assays may include amplification of target sequences by any of several amplification techniques, for example, PCR, NASBA or TMA. Amplification assays may be monitored in real time utilizing intercalating dyes, for example SYBR green, or fluorescently labeled probes, such as 5' nuclease probes Livak, K.J. et al. (1995), Oligonucleotides with fluorescent Dyes at Opposite Ends Provide a Quenched Probe System Useful for Detecting PCR Product and Nucleic Acid Hybridization, PCR Meth. Appl. 4: 357-362, dual FRET probes, Espy, M.J. et al. (2002), Detection of Vaccinia Virus, Herpes Simplex Virus, Varicella-Zoster Virus, and Bacillus anthracis by LightCycler Polymerase Chain Reaction after Autoclaving: Implications for Biosafety of Bioterrorism Agents, Mayo Clin. Proc. 77: 624-628, or molecular beacon probes, Tyagi, S. and Kramer, F.R. (1996), Molecular Beacons: Probes that Fluoresce upon Hybridization, Nature Biotechnol. 14: 303-308; Tyagi, S. et al. (1998), Multicolor Molecular Beacons for Allele Discrimination, Nature Biotechnol. 16: 49-53. Real-time multiplex assays utilizing PCR amplification have been demonstrated with TaqMan dual-labeled linear probes and the 5' nuclease detection process and, alternatively, with PCR amplification and molecular beacon probes, Tyagi. S. *et al.* (1998), *supra*; Vet, J.A. et al. (1999), Multiplex Detection of Four Pathogenic Retroviruses Using Molecular Beacons, Proc. Natl. Acad. Sci. USA 96: 6394-6399; El-Hajj, H. et al. (2001), Detection of Rifampin Resistance in Mycobacterium tuberculosis in a Single Tube with Molecular Beacons, J. Clin. Microbiol. 39: 4131-4137. Fluorescence-based multiplex assays currently are limited to about eight targets per sample by the need to minimize overlaps in emission spectra of fluorophores and, hence, are not expandable for use as highly multiplexed screening assays.

[0003] Highly multiplexed assays rely on spatial segregation of targets for signal resolution. Spatial segregation enables the use of coding schemes involving combinations of differently colored fluorophores (combinatorial coding), combinations of different amounts of each fluorophore (ratio coding), and both. One example of an assay with spatial segregation is fluorescence *in situ* hybridization, or FISH, for chromosomal analysis. Speicher et al. (1996), Karyotyping Human Chromosomes by Combinatorial Multi-fluor FISH, Nature Genet. 12: 368-375, for example, report the use of 27 probes combinatorially labeled using a set of six different fluorophores for analysis of chromosome spreads. Similarly, segregation of transcription sites in cell nuclei has enabled combinatorial coding utilizing multiple, singly labeled probes per site, as well as ratio coding. Singer, R.H., International (PCT) patent application WO 00/65094; Levsky, J.M. et al. (2002), Single-Cell Gene Expression Profiling, Science 297: 836-840. Another spatial-segregation probe technique is the use of multiplex probe arrays, including arrays on DNA chips. Schena, M. et al. (1995), Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray, Science 20: 467-470; Gingeras, T.R. et al. (1998), Simultaneous Genotyping and Species Identification Using Hybridization Pattern Recognition Analysis of Generic Mycobacterium DNA Arrays. Genet. Res. 8: 435-448; Han et al. (2001), Quantum-Dot-Tagged Microbeads for Multiplexed Optical Coding of Biomolecules, Nature Biotechnol. 19,631-635. Another segregation approach is the use if electrophoresis to separate ligated probe pairs of differing lengths. Tong, A.K. et al. (2001), Combinatorial Fluorescence Energy Transfer Tags for Multiplex Biological Assays, Nature Biotechnol. 19: 756-759. In that method, a variation of the oligonucleotide ligation assay ("OLA") for SNP detection, differently labeled probes are ligated to capture probes on a target, and the hybrids are immobilized, washed, released, separated from one another by electrophoresis, and read for each probes' fluorescent code, which is a combination of colors and ratios. Array methods and electrophoretic methods remain technically complex, requiring many separate steps, including amplification, hybridization, washing and analysis.

[0004] WO-A-03/020967 disclosed a method for determining at least three analytes, wherein one probe has a first label, a second probe has a second label, and a third probe has two sub-populations: one having the first label, the other having the second label. The analytes are detected according to the signal intensities determined for the first and second labels. The system may be extended to more analytes as exemplified by a six analyte system using three labels.

[0005] There is not currently available a homogeneous fluorescence hybridization assay that is suitable for use as a highly multiplexed screening assay, despite the need for such an assay. During suppressive treatment

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of the HIV-1 virus with protease inhibitors, for example, any of about 30 mutations is likely to proliferate over time and to require change in treatment. Hirsch, M.S. et al. (1998), Antiretroviral Drug Resistance testing in Adults with HIV Infection, JAMA 279: 1984-1991. Lacking a highly multiplexed screening assay, current practice is to sequence the virus in response to a patient's increase in viral load. Sequencing is made difficult by the fact that the arising mutant is not the only allele present. During initial diagnosis of a patient with particular symptoms, for example fever, there is available no highly multiplexed homogeneous assay to screen for an early indication of one of numerous possible infectious agents that may be the cause of the patient's symptoms.

[0006] An aspect of this invention is highly multiplexed homogeneous assays for screening samples for the presence of at least six target nucleic acid sequences, utilizing conventional fluorescence detection equipment and techniques, and fluorescently labeled hybridization probes.

[0007] Another aspect of this invention is kits for carrying out particular screening assays according to this invention.

SUMMARY

[0008] Assays according to this invention are highly multiplexed assays suitable for screening. By "highly multiplexed," I mean that an assay is capable of detecting any of at least six, preferably at least 10, and in certain embodiments 30-60 and even more possible target sequences.

[0009] Assays according to this invention are homogeneous nucleic acid *in vitro* assays fluorescently labeled utilizing hybridization probes. By "homogeneous", I mean that nucleic acid targets are detected in solution without segregating targets spatially and without washing away unbound hybridization probes. Fluorescence signal is obtained from the reaction mixture itself. Certain preferred embodiments include target amplification by, for example PCR or NASBA, which enables detection of small amounts of pathogens in samples. When amplification is used, the reaction vessel, for example a microcentrifuge tube, is hermetically sealed to prevent contamination of the other samples being tested at the same time, samples not yet tested, equipment and workers.

[0010] Detection in assays according to this invention is by fluorescently labeled nucleic acid hybridization probes. Fluorescent labels, when stimulated emit radiation in the visible, ultraviolet or near infrared portions of the electromagnetic spectrum. Each probe is specific to one target among the multitude of possible targets being screened. If the goal and design of an assay are to distinguish among bacterial species, one will design a probe for a species, for example against a conserved region of *M. tuberculosis,* or design a probe that hybridizes to nonidentical sequences of multiple strains of the same species. If the goal, on the other hand, is to distinguish among strains or mutant alleles, one will design each probe against a variable region to provide the necessary distinction and make the probe strain-specific. Preferred probes for use in this invention are molecular beacon probes, which may be made mismatch tolerant or mismatch intolerant, depending on the need of a particular assay. As is known in the art, choice of target sequence and probe design are balanced to minimize both false negatives and false positives.

[0011] Multiplex capability is achieved by combinatorial coding, with or preferably without added ratio coding, utilizing a panel of distinguishable fluorophores. In a combinatorial code, each code element is identified by its unique pattern of different labels, in this case colors. The combinatorial code includes up to four different fluorophores per code element. The different fluorophores are from a panel of at least four distinguishable fluorophores, more preferably a panel of 4-8 distinguishable fluorophores. The combinatorial code may be duplex coding in which the code elements comprise two colors, optionally enlarged by additional code elements of one color. The combinatorial code may by triplex coding in which the code elements comprise three colors, optionally enlarged by additional code elements of two colors (triplex code combined with a duplex code), and optionally enlarged by code elements of one color. The combinatorial code may be quadraplex coding in which the code elements comprise four colors, optionally enlarged by additional code elements of three colors, two colors or one color, or some combination thereof.

[0012] Rather than labeling individual probe molecules with multiplex colors to give the probe the pattern assigned to it as a code element, each probe is subdivided into the required number of portions, and each portion is labeled to emit a different color signal. The portions are then mixed to create a probe that, when hybridized to its target, will include the unique color signal pattern of the code element.

[0013] For the preferred highly quenched probes such as molecular beacon probes, each portion will emit a single color. For less preferred probes labeled with two different interacting fluorophores, such as 5'-nuclease probes, one option is to utilize a common short-wavelength fluorophore (which I call the "harvester" fluorophore), vary the long-wavelength fluorophores (which I call the "emitter" fluorophores), and detect the signal change in the latter. In that case, each portion will emit a signal in a different color. Another option is the reverse, namely, to use a common emitter fluorophore, vary the harvester fluorophores, and detect the signal change in the latter. In that case, each portion will also emit a signal in a different color, but for purposes of coding it is the harvester that becomes the detected emitter. However, a third option is to vary both fluorophores to permit detection of a ratio change in their emissions, so that the signal from one portion might be the ration a/b, the ratio from a second portion might be the ratio c/d, and the ratio from a third portion might be e/f, where a, b, c, d, e and

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f are distinguishable fluorophores. In this case the long-wavelength fluorophores are varied and can be considered to characterize the signal one obtains from any given probe portion. For ease of understanding, descriptions of combinatorial codes in this application and in the figures generally describe embodiments in which one detects for each probe portion the emission of a particular color, which can be characterized as the emitter fluorophore. It is to be understood that the teachings also apply to embodiments utilizing ratios, and persons skilled in the art will be able to apply the teachings to those embodiments.

[0014] If one begins with a panel of eight different fluorophores, for example, and chooses to subdivide each probe into four portions and to label each of the four portions with a different color, the codebook can include as many as 70 different combinations; if one begins with the same-sized panel of fluorophores but subdivides each probe into three portions and labels each of the three portions with a different color, the codebook can include as many as 56 different combinations; and if one begins with the same-sized panel of fluorophores but subdivides each probe into two portions and labels each of the two portions with a different color, the codebook can include as many as 28 different combinations. Four-color, three-color and two-color codes may be combined with each other and even with a one-color code, but my most preferred embodiments do not include such combinations. In ratio coding differing amounts of the same label are used for differentiation. Combinatorial codes according to this invention may be augmented with ratio coding, preferably integral ratio coding, that is, ratios of whole numbers such as 2:1, 3:1, 4:1 or 5:1, and vice versa. It is preferred that portions of molecular beacon probes be labeled with a non-fluorescent quencher and one signature-color fluorophore, most preferably without added ratio coding for reasons that will be explained.

[0015] As stated above, assays according to this invention are homogeneous detection assays. There is no segregation of targets. There is no separation of unbound probes from probes bound to their targets. Consequently, in assays according to this invention there must be a detectable signal change indicative of a probe hybridizing to its target. Preferred molecular beacon probes are highly quenched when free-floating at the detection temperature in the assay but change conformation and fluoresce when hybridized to their respective targets. Another type of probe whose fluorescence is restored by hybridization to its target and that is generally suitable for use in assays according to this invention is a "yin-yang" probe, a bimolecular probe labeled with a fluorophore on a target complementary strand and a quencher on a competing strand. Li, Q. et al. (2002), A New Class of Homogeneous Nucleic Acid Probes Based on Specific Displacement Hybridization, Nucleic Acids Res. 30: e5. Other probe-assay schemes result in a fluorescent signal change indicative of probes hybridizing to targets. In the 5'-nuclease detection process, end-labeled linear (or random-coil) probes with two different fluorophores interact by fluorescence resonance energy transfer (FRET) when free-floating but are cleaved during primer extension in a polymerase chain reaction (PCR) amplification, resulting in a signal change (emission from the shorter wavelength fluorophore increases, emission from the longer wavelength fluorophore decreases, and the ratio of the two changes accordingly). Livak, K.J. *et al.* (1995), *supra.* Another system, so-called LightCycler probes, works in the opposite manner. Each probe is a pair of fluorescently labeled linear oligonucleotides that hybridize adjacently on a target and interact by FRET, thereby producing a signal change (emission from the shorter wavelength fluorophore decreases, emission from the longer wavelength fluorophore increases, and the ratio of the two changes accordingly). Espy, M.J. *et al.* (2002), *supra.* For FRET systems, I refer to the shorter wavelength fluorophore as a "harvester" and the longer wavelength fluorophore as an "emitter". However, because the background resulting from FRET quenching is considerably higher than contact-mediated quenching that is commonly used in molecular beacon probes and yin-yang probes (Tyagi *et al.* (1998), *supra;* Marras, S.A.E. et al. (2002), Efficiencies of Fluorescence Resonance Energy Transfer and Contact-Mediated Quenching in Oligonucleotide Probes, Nucleic Acids Res. 30: e122), FRET quenching is not preferred for screening assays according to this invention. Assays according to this invention preferably employ probes that are quenched by contact-mediated quenching, such as molecular beacon probes or yin-yang probes. My most preferred probes are molecular beacon probes that include a non-fluorescent quencher. These probes are most preferred, not only because of their low background signal, but also because of their ease of design for a multiplex system. Bonnet, G. et al. (1998), Thermodynamic Basis of the Enhanced Specificity of Structured DNA Probes, Proc. Natl. Acad. Sci. USA 96: 6171-6176.

[0016] Assays according to this invention include, but are not limited to, embodiments that employ target amplification. Several exponential amplification techniques are well known. Among them are the polymerase chain reaction (PCR), which includes thermal cycling, and isothermal amplification schemes such as nucleic acid sequence-based amplification (NASBA), strand-displacement amplification (SDA), transcription-mediated amplification (TMA), rolling-circle amplification (RCA), and ramification amplification methodology (RAM). Amplification assay embodiments may utilize end-point detection or real-time detection. It is preferred to perform amplification in a sealed environment, for example sealed tubes, in the presence of the hybridization probes to minimize the possibility of cross contamination between samples that results from opening sample tubes.

[0017] While not limited by sample source, assays according to this invention advantageously may utilize human or animal samples sometimes referred to as being normally sterile, such as blood, tissue or spinal fluid. Such

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samples are advantageous as compared, for example, to sputum, stool or environmental samples, because they will contain fewer extraneous DNA sequences that could potentially interfere with amplification and detection. It is known that at least small amounts of many pathogens can be found in a patient's blood, even in septic syndromes resulting from bacterial infections. It is preferred to utilize normally sterile sources rather than non-sterile sources where a particular screening assay permits a choice.

[0018] Certain amplification assays according to this invention may advantageously take advantage of primers that amplify all or a number of nucleic acid targets being screened. When screening for mutations, it may be that numerous possible mutations occur in a particular variable nucleic acid sequence of interest, and one pair of primers flanking that region can be used to amplify all such possible mutations. Certain of these so-called "universal primers" bind to conserved regions of multiple species and are used for amplifying numerous targets, for example genes of various bacteria. See, for example, Kox, L.F.F. et al. (1995), PCR Assay Based on DNA Coding for 16S rRNA for Detection and Identification of Mycobacteria in Clinical Samples. J. Clin. Microbiol. 33: 3225-3233; Iwen, P.C. et al. (1995), Evaluation of Nucleic Acid-Based Test (PACE 2C) for Simultaneous detection of Chlamydia trachomatis and Neisseria gonorrhoeae in Endocervical Specimens, J. Clin. Microbiol. 33: 2587-2591; Yang, S. et al. (2002), Quantitative Multiprobe PCR Assay for Simultaneous Detection and Identification to Species Level of Bacterial Pathogens, J. Clin. Microbiol. 40: 3449-3454; Schonhuber, W. et al. (2001), Utilization of mRNA Sequences for Bacterial Identification, BMC Microbiol. 1: 2; Wong, R.S. and Chow, A.W. (2002), Identification of Enteric Pathogens by Heat Shock Protein 60kDa (HSP 60) Gene Sequences, FEMS Microbiol. Lett. 206: 107-113. One pair, more likely two or three pairs, of primers may suffice for particular screening assays.

[0019] This invention includes assay kits for performing particular multiplex screening assays according to this invention. Kits include at least the intended complement of detection probes. We refer to collections of oligonucleotides as "oligonucleotide sets." When the assay is an amplification assay, for example a PCR assay, the oligonucleotide sets preferably also include the primers needed for amplification. They may also include any amplifiable control sequence. Kits may include additional assay reagents such as enzymes, amplification buffer and, if desired, sample preparation reagents for isolation of nucleic acids; that is, up to all reagents needed for performing the assay, from sample preparation through detection.

[0020] Fluorescence-based screening assays of this invention are generally adaptable to detection instruments, which may include thermal cycling capability. Instruments may use a single-wavelength source or a multi-wavelength source, for example a white light combined with selectable filters, multiple laser sources or multiple light-emitting diode sources. Detection instruments vary in their ability to distinguish emissions from multiple fluorophores, which needs to be taken into account when selecting fluorophore panels for a particular assay. In preferred embodiments of assays according to this invention, emissions from fluorophores for each probe are balanced in intensity such that intensities from the subdivided portions are closely spaced, which aids identification. Fluorophores vary in intensity, target sequences vary in accessibility for probe hybridization, and instruments may vary in their responses to different wavelengths. Relative amounts of portions may be varied to achieve balance. Where possible, it is preferred that all portions for all probes be balanced to further aid in identification. The results of these assays can be automatically decoded and interpreted by computer programs in real time, enabling the assays to be highly accurate, and to be carried out in an automated, high-throughput fashion in clinical diagnostic settings.

[0021] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

[0022]

FIG 1 is a table showing the numbers of distinguishable combinations obtained using two or three colors per probe and panels of 4-8 distinguishable fluorophores.

FIG 2 illustrates fluorescence emissions of a real-time amplification assay in which a single target sequence is present in the sample being tested (out of many possible target sequences that the assay is designed to detect) and one probe (labeled with three different colors of equal fluorescence intensity) among the many different probes in the assay mixture binds to the amplified target sequence.

FIG 3 illustrates fluorescence emissions of a real-time amplification assay in which two different target sequences are present at different concentrations in the sample being tested and two probes (each labeled with a different combination of three different colors of equal fluorescence intensity, no color being shared by both probes) bind to the amplified target sequences.

FIG 4 illustrates fluorescence emissions of a real-time amplification assay in which two different target sequences are present at different concentrations in the sample being tested and two probes (each labeled with a different combination of three different

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colors of equal fluorescence intensity, one color being shared by both probes) bind to the amplified target sequences.

FIG. 5 illustrates fluorescence emissions of a real-time amplification assay in which two different target sequences are present at the same concentration in a sample being tested, and two probes (labeled according to the first ternary color codebook shown in Fig. 6a) share two colors, leading to unambiguous interpretation of the results.

FIGS. 6a and 6b contain tables that show the effect of alternative probe coding schemes for detecting ten different possible targets in a sample, utilizing probes that are each labeled with three different colors of equal fluorescence intensity from a palette of five different fluorophores, on the results that would be obtained for every one of the 45 possible combinations of two different targets that could be present at the same concentration in a sample being tested.

FIG. 7 illustrates fluorescence emissions of a real-time amplification assay in which two different target sequences are present at the same concentration in a sample being tested, and two probes (labeled according to the first ternary color codebook shown in Fig. 6a) share one color, leading to an ambiguous result.

FIG 8 illustrates fluorescence emissions of a real-time amplification assay in which the same sample used in the assay shown in Fig. 7 is retested using probes labeled according to the second ternary color codebook shown in Fig. 6b, providing an example of how a second assay utilizing an alternative coding scheme can resolve ambiguous results.

DETAILED DESCRIPTION

[0023] FIG 1 is a table presenting certain combinatorial coding schemes useful in this invention. The top portion of the table presents the number of codes obtained by having each probe differently labeled with two different fluorophores from panels of 4, 5, 6, 7 or 8 fluorophores. If the panel includes eight fluorophores, there are 28 possible codes. The bottom portion of the table presents the number of codes obtained by having each probe labeled with three different fluorophores from the same panels of 5, 6, 7 or 8 fluorophores. If the panel includes eight fluorophores, there are 56 possibilities. Assays according to this invention utilize two or three (and sometimes four) differently labeled portions of each probe and a panel of fluorophores of sufficient size to permit at least six differently coded probes. This may be accomplished, for example, by combining the two portions of the table, that is, using both codes of two colors and codes of three colors. For example, probes may be subdivided into three portions for use with a panel of five fluorophores, providing up to ten differently coded probes, as outlined in the bottom portion of the table, and additional probes may be subdivided into two portions for use with a panel of five fluorophores, providing up to ten additional differently coded probes, as outlined in the top portion of the table. In this manner, as many as 20 differently coded probes can be combined in the same assay mixture.

[0024] Assays according to this invention utilize one probe (which may be a singlemolecule probe, such as a molecular beacon probe or a TaqMan probe, or a bi-molecular probe, such as a yin-yang probe or a LightCycler probe pair) for each target. Coding is achieved, except for a code element represented by a single color, by subdividing probes into portions and labeling the portions according to a combinatorial code or combinatorial-plus-ratio code from a panel of at least four, preferably 5-8, distinguishable fluorophores. Utilizing our preferred molecular beacon probes for purposes of illustration, each probe portion is designed to have a different color signature. Certain of the probes may be wavelength-shifting probes, that is, probes in which one arm is labeled with a quencher and the other arm is labeled with a FRET pair comprising a "harvester" fluorophore to absorb energy from the stimulating light source and transfer that energy to an "emitter" fluorophore that fluoresces strongly at its own characteristic longer wavelength spectrum when the probe is in its open configuration. We utilize wavelength-shifting probes with instruments that employ a single wavelength source that is not highly absorbed by fluorophores that emit visible fluorescence at longer wavelengths, for example, Texas Red. See Tyagi, S. et al. (2000), Wavelength-Shifting Molecular Beacons, Nature Biotechnol. 18: 1191-1196; El-Hajj, H. *et al.* (2001), *supra.* It is also possible to utilize one fluorophore to quench another fluorophore by contact-mediated quenching. This creates two additional possibilities. First, a fluorophore that has relatively low emission in the system being used could be placed on each arm so that the fluorophores quench one another when the probe is closed but both fluoresce with the same color, increasing its intensity, when the probe is hybridized to its target and open. This technique can be used to add ratio coding to a combinatorial code. Second, one portion of a probe can carry two code elements (colors) by placing different fluorophores on the two arms, so that the fluorophores quench one another when the probe is closed but each fluoresces in its own characteristic emission spectrum when the probe is hybridized to its target and open. This can be used to combine two portions into one and thereby reduce the number of different portions needed for a two-color, three-color or four-color code element. It is equivalent to having separate portions. Because quenching by non-fluorescent quenchers is more efficient than quenching by fluorophores and because having two emitting fluorophores on the same molecule removes the option to balance their intensities by means of relative con-

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centration, preferred embodiments of this invention utilize a non-fluorescent quencher on one arm of molecular beacon probes. Dabcyl is a preferred quencher, as is Black Hole Quencher No. 2.

[0025] "Codebooks" useful in this invention utilize four colors per probe, three colors per probe, two colors per probe, or combinations of four, three or two colors per probe, with or without single-color probes. Preferred codebooks utilize either only four colors per probe, only three colors per probe or only two colors per probe. The reason these are preferred is that the appearance of, for example, one color arising at a particular PCR cycle signals that an error has been made in assay preparation. If single-color probes are used, such an error is not obvious. In some embodiments of this invention, combinatorial coding may be supplemented with ratio coding to increase the number of code elements or possibilities. Preferred ratios are integral, from 5:1 to 1:5, preferably from 3:1 to 1:3. Ratio coding is most appropriate for screening assays in which it is highly likely that only one target will be found. The reason for this is that when the code is strictly combinatorial and two or more targets are present, changes in intensity levels are helpful for resolving ambiguities that may arise in analysis of the emission spectrum. Therefore, preferred assays that are not highly likely to find only one target utilize strictly combinatorial coding.

[0026] Various fluorophores can be included in panels of fluorophores. Certain fluorophores have relatively narrow emission spectra, for example, Vic, Alexa 488, and quantum dots (Gao et al. (2002), Quantum-Dot Nanocrystals for Ultrasensitive Biological Labeling and Multicolor Optical Encoding, J. Biomed. Opt. 7, 532-537), making them particularly useful for highly multiplexed assays utilizing panels of more than six fluorophores.

[0027] Oligonucleotide probes, including molecular beacon probes, are conveniently prepared by solid-phase DNA synthesis on, for example, an Applied Biosystems 394 DNA/RNA synthesizer. DNA nucleotides or RNA nucleotides may be used, including modified nucleotides such as 2'-O-methylribonucleotides, which yield strands resistant to cleavage. Our preferred method of molecular beacon synthesis starts with controlled-pore glass columns containing the quencher dabcyl for incorporation at the 3' end of the probe. These columns are available from Biosearch Technologies (Novato, California, USA). Our preferred method of incorporating fluorophores at the 5' end of the probe is to utilize thiolmodifier phosphoramidites or aminomodifier phosphoramidites so that iodoacetylated fluorophore derivatives can be coupled to the 5'-thiol groups and succinimidyl esters of fluorophores can be coupled to the 5'-amino groups. For tetrachlorofluorescein, our practice is to incorporate directly a tetrachlorofluorescein phosphoramidite. For wavelength-shifting probes a non-terminal fluorophore is required. We have used fluorescein phosphoramidites to incorporate internal fluorescein moieties for this purpose. Our molecular beacon probe synthesis includes probe purification by gel exclusion chromatography through NAP-5 Sephadex columns, followed by HPLC through a reverse-phase column such as the C-18 column marketed by Waters. Finally, probes are precipitated with ethanol and dissolved in 100 μl Tris-EDTA buffer. Molecular beacon probes should have a signal-to-noise ratio of at least 25, preferably closer to 100, keeping in mind that for a triplet code, for example, each fluorophore will be common to 30-60% of the probes.

[0028] As indicated above, the probes are subdivided into two, three or four portions for labeling and use. It is advantageous that the various portions of a probe, and where possible all portions in the assay, produce roughly the same signal intensity. Accordingly, our preferred assays and kits have portions "balanced" for that purpose. By "balanced" I mean that the fluorescence intensities of the signals from the different portions are generally within 20% of one another, most preferably within 5%. The fluorescence intensity produced by a particular probe portion, for example a molecular beacon probe portion, will depend on the intrinsic properties of its emitter fluorophore (and, if it is a wavelength-shifting molecular beacon probe, also its harvester fluorophore), the nature of the target, as some targets accept probes more readily than others, and quite possibly on the instrument utilized for detection. By testing a probe against its target in the assay on the instrument to be used, the amounts of the portions relative to one another can be adjusted to produce balanced signals. I prefer further that all portions of all of the combinatorially labeled probes be balanced. After the portions of each probe are balanced, the amounts of the probes relative to one another can be adjusted to produce balanced signals. It is possible to adjust signal readings by adjusting the detection instrument rather than the amounts of the portions of each probe to account for differences in the fluorophores and the instrument. For example, if a probe with three portions labeled a, b, c produces relative intensities with equal volumes of each portion of 1.0, 1.5 and 3.0, respectively, the instrument could be programmed to multiply readings from portion "a" by three, from portion "b" by two, and from portion "c" by one. Then only the relative amounts of the probes would have to be balanced. My preference, however, is to adjust the portion amounts rather than the instrument. Utilizing balanced emissions makes interpretation by a human observer significantly easier, as one can see immediately whether or not three colors, for example, indicate a particular code element.

[0029] Assays according to this invention are highly multiplexed. While not limited as to sample source, the assays are particularly applicable for use with samples from human or other animal body sites that are considered sterile, such as blood, tissue and cerebral spinal fluid. Non-sterile specimens, such as sputum or stool, are likely to have nucleic acids from a variety of sources and are more difficult to screen for that reason. Pathogens generally infect the blood stream to at least a small degree. However, nucleic acid amplification assays re-

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quire minimal amounts of target sequence in the sample. For example, Kane *et al.* compared blood culturing to PCR assays utilizing "universal' bacterial primers to diagnose bacterial infection in critically ill surgical patients. Kane, T.D. et al. (1998), The Detection of Microbial DNA in Blood: a Sensitive Method for Diagnosing Bacteremia and/or Bacterial Translocation in Surgical Patients. Ann. Surg. 227: 1-9. Whereas blood cultures were positive for only 14% of the samples, the PCR assays were positive for 64%.

[0030] For embodiments employing amplification, such as PCR or NASBA, primers are required for the various possible targets. Primer design is known in the art. Primers can be tested with other primers and then with probes to check for undesired interactions. It is important that primers, and to a lesser extent probes, not interact in multiplex reactions. A type of high-specificity primer useful in this invention is disclosed in published international patent application WO 00/71562. Such primers form hairpin stems that significantly reduce undesired hybridizations. We have decreased primer interactions by utilizing such high-specificity primers, taking care to ensure that the melting temperature (T_m) of the stem is approximately 6°C above the primer annealing temperature utilized in the assay. We have constructed two pairs of universal primers that together amplify a segment of the 16s ribosomal RNA gene of many bacterial species. One pair includes 5'-TGACGACAACCATGCACC-3' (SEQ. ID NO. 1) and 5'-ATGTGGTTTAATTCGAAGCAA-3' (SEQ. ID NO. 2) to amplify target regions in *B. anthracis* and related gram-positive bacterial. The other pair includes 5'-GTGGACTTAGATACCCTGGTAGTCCAC-3' (SEQ. ID NO. 3) and 5'-GCGTTGCATCGAATTAA-3' (SEQ. ID NO. 4), which amplify target regions in several important gram-positive bacteria and gram-negative bacteria. The underlined sequences of the third identified probe are hairpin-forming sequences added to reduce undesired hybridizations by this primer.

[0031] Amplification assays with real-time detection can be performed on a variety of instruments that utilize different techniques for exciting fluorophores and detecting emissions. Excitation can be by lasers, white light coupled with filters, or light-emitting diodes. In addition, the assay instrument may utilize a synchronous scanning mode, in which narrow excitation and emission wavelengths are continuously varied, while maintaining a fixed wavelength difference between them. Lee et al. (1999), Seven-Color, Homogeneous Detection of Six PCR Products, Biotechniques 27, 342-349. The choice of instrumentation may affect the number of different fluorophores that can be included in a panel.

[0032] Screening assays according to this invention, including but not limited to real-time amplification assays, may be positive for a single target, in which case the target is identifiable by its unique signature, preferably its unique color signature but possibly by its color-plus-intensity signature. On occasion two or even more than two targets may be present in a sample. Deciphering the spectrum in such a case may take advantage of the fact that, although the absolute fluorescence of a probe is dependent on target concentration, the fluorescence ratios of two probe portions hybridizing to the same target will be independent of target concentration. Taking the case of strictly combinatorial coding utilizing three-color codes wherein the intensities of the portions have been balanced, assume that two targets are present. If the targets share no color, there will result six colors. However, because equal concentrations of the two targets in the sample are highly unlikely, direct probing of the sample is likely to result in three colors of one intensity and three colors of a second intensity, thereby identifying the two targets. If the assay, on the other hand, is a real-time amplification assay, the times at which signals rise above background, for example the threshold cycle (C_T) of a PCR reaction, are likely to differ, leading to two groups of three colors, each group arising at a different time, thereby identifying the two targets.

[0033] If the codes assigned to the probes for the two targets share a color, in an assay based on directly probing the nucleic acids in a sample, the fluorescence intensity for that color will not match the fluorescence intensity for the other colors. Rather it will be a combination from two targets and will stand out from the level of the other colors, thereby identifying it as the color in common. If the assay, on the other hand, is a real-time amplification assay, three colors will come up together as signal from the more abundant target. When the second threshold for the less abundant target is reached, two more colors will come up together and the intensity curve for the common color will change slope to a higher value, thereby identifying it as the color in common.

[0034] Taking the case where ratio coding is also used, assume one color is common to two targets, but its intensity ratio for the more abundant target is 2:1 as compared to the other portions for that target. In a real-time amplification assay three colors will rise above background as the first threshold is reached, but one color (the common color) will have a higher slope than the other two colors. Nonetheless, when the second threshold is reached, the intensity curve of the common color will change slope to a higher value, signifying its commonality.

[0035] When two different target species are present in the sample at the same concentration, the colors encoding each probe will appear at the same time. In some instances of this situation it may not be possible to unambiguously identify each code. In such an instance a repeat assay utilizing an alternative coding scheme for the targets, should in almost all cases resolve the ambiguity.

Example 1

[0036] In this example, a real-time PCR amplification assay is carried out on DNA isolated from a normally

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sterile blood sample suspected of containing an infectious bacterium. The sets of primers that are present in the reaction mixture enable the amplification of a segment of the 16s ribosomal RNA gene that is present in all of the bacterial species that are suspected of possibly being present in the blood sample. A multiplicity of molecular beacon probes is present in the assay, one for each of the suspected bacterial target species. Each different molecular beacon possesses a universal quencher moiety (dabcyl) covalently linked to its 3' end. Each different species-specific molecular beacon probe is labeled combinatorially according to this invention with a unique set of three differently colored fluorophores. These fluorophores are covalently linked to the 5' end of each oligonucleotide. For every species-specific probe that is present in the assay mixture, the abundance of each of the three differently colored oligonucleotides that together make up the species-specific probe is adjusted prior to performing the assay so that the fluorescence intensity of each of the three colors of that probe will be approximately the same when measured in the assay instrument used to carry out the assay. Fig. 2 is a plot of results that will be obtained in this assay. The "x" axis represents the number of amplification cycles performed by the time that each measurement of fluorescence is made. This measurement is made automatically by the assay instrument in a manner that permits the fluorescence intensity of each of the differently colored fluorophores that are used to label the probes to be measured. The "y" axis represents the fluorescence intensity due a particular colored fluorophore. Measurements are taken during the annealing phase of each amplification cycle. Measurements are taken in real time during each amplification cycle throughout the course of the amplification. The fluorescence intensity of each of the differently colored fluorophores used to label the probes is plotted as a function of the number of amplification cycles completed.

[0037] In the experiment shown in Fig, 2, a signal appears that is sufficiently intense to be detected above the background fluorescence after a particular number of cycles of amplification have been carried out. The amplification cycle at which this occurs is known as the "threshold cycle", generally abbreviated as "C_T". The signal that appears after the threshold cycle consists of three different colors (a, b, and c). This signal rises in a linear fashion as more cycles of amplification are completed. The set of three colors that appear together in combination identify which bacterial species is present in the blood sample. The abundance of the species in the sample is determined from the threshold cycle at which the signal arises. The threshold cycle is inversely proportional to the logarithm of the number of target molecules in the sample

Example 2

[0038] In this example, an assay is carried out in the same manner and for the same purpose as the assay described in Example 1. The results that will be obtained in this experiment are shown in Fig. 3. Two different signals occur, one (consisting of colors a, b, and c) appears early in the reaction, and the other (consisting of colors d, e, and f) appears later in the reaction. This result indicates that two different bacterial species are present in the sample, one (identified by colors a, b, and c) is relatively abundant, and the other (identified by colors d, e, and f) is relatively rare and therefore requires more cycles of amplification before there is sufficient amplified product for the fluorescence signal due to the hybridization of the species-specific probe to be seen above the background fluorescence. The unique set of colors in each of the two signals unambiguously identifies each of the two bacterial species that are present in the sample.

Example 3

[0039] In this example, an assay is carried out in the same manner and for the same purpose as the assay described in Example 1. The results that will be obtained in this experiment are shown in Fig. 4. Two different signals occur, one (consisting of colors a, b, and c) appears early in the reaction, and the other (consisting of colors d and e) appears later in the reaction. In addition, the slope of the curve showing the fluorescence intensity of color "c" increases after the threshold cycle at which the later signal (consisting of colors d and e) occurs. This result indicates that two different bacterial species are present in the sample, one (identified by colors a, b, and c) is relatively abundant, and the other (identified by colors c, d, and e) is relatively rare and appears later. The increase in the slope of the curve showing the fluorescence intensity of color "c" is due the contributions of "c" fluorescence from probes binding to amplified segments of the DNA from the rare bacterial species being added the "c" fluorescence from probes binding to amplified segments of the DNA from the abundant bacterial species. Even though the three-color codes for each of the two bacterial species that are present in the sample have one color in common, the occurrence of the two species at different concentrations in the sample enables the signals from each species to be distinguished from one another. In general, when two (or even three) species are simultaneously present in a clinical sample, each will occur at a different concentration, and the coded signal due to the presence of each of the bacterial species can be distinguished from the signals due the other species in the sample.

Example 4

[0040] This example illustrates how unambiguous results can be obtained even though two different species are present in a sample at approximately the same concentration. The assay described in this example is carried out in the same manner and for the same purpose as the assay described in Example 1. In this experiment, the probes present in the assay mixture are designed to de-

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tect ten different bacterial species (designated species 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9). Three-color codes are used to label each of the ten probes, from a palette of five differently colored fluorophores (designated a, b, c, d, and e). For every species-specific probe that is present in the assay mixture, the abundance of each of the three differently colored oligonucleotides that together make up the species-specific probe is adjusted prior to performing the assay so that the fluorescence intensity of each of the three colors of that probe will be approximately the same when measured in the assay instrument used to carry out the assay. For this assay, the following coding scheme (which I call the "first ternary color codebook") is used:

probe for species 0	abc
probe for species 1	abd
probe for species 2	abe
probe for species 3	acd
probe for species 4	ace
probe for species 5	ade
probe for species 6	bcd
probe for species 7	bce
probe for species 8	bde
probe for species 9	cde

[0041] The results that will be obtained in the experiment carried out with probes labeled according to this codebook are shown in Fig. 5. A signal consisting of four different colors (a, b, c, and d) arises at the same threshold cycle. The slope of the curves for two of the colors (a and b) is twice as high as the slope of the curves for the other two colors (c and d). This result indicates that two different bacterial species are present in the sample at approximately the same concentration, and that the three-color codes of their respective probes have two colors in common. Since the slope of the curves for colors "a" and "b" is approximately twice as high as the slope of the curves for colors "c" and "e", colors "a" and "b" are components of the codes for both bacterial species that are present in the sample. The conclusion to be drawn from these results is that one of the probes that bound to amplified target sequences was encoded "abc" and the other probe that bound to amplified target sequences was encoded "abd". By comparing these results with the coding scheme in the first ternary color codebook (shown above), "abc" is seen to be the code for bacterial species "0" and "abd" is seen to be the code for bacterial species "1". Thus, the results unambiguously indicate that the sample contains species "0" and species "1" in approximately equal concentration.

Example 5

[0042] This example illustrates how ambiguous results can sometimes be obtained when two different species are present in a sample at approximately the same concentration. The assay described in this example is carried out in the same manner and for the same purpose as the assay described in Example 4. The probes used in this assay utilize the first ternary color codebook (shown in Example 4).

[0043] Fig. 6a contains a two-part table (based on the first ternary color codebook) that shows the results that should occur when two different species are present in a sample at the same concentration for each of the 45 combinations of two species that are possible. In 30 of these combinations four colors arise, two at a higher rate of fluorescence intensity increase (identified by underlined letters) and two at a lower rate of fluorescence intensity increase (identified by letters that are not underlined). Each of these 30 combinations is unique within the table, therefore providing an unambiguous result.

[0044] However, from an examination of Fig. 6a it can be seen that in 15 of the 45 possible combinations of two different species that can be present in a sample at the same concentration five colors arise, one at a higher rate of fluorescence intensity increase (identified by an underlined letter) and four at a lower rate of fluorescence intensity increase (identified by letters that are not underlined). These combinations, however, are not unique. For example, the code "abcde" occurs three times in the upper table: once for the combination of species "2" and "9", a second time for the combination of species "4" and "8", and a third time for the combination of species "5" and "7". An example of an experiment that yields such an ambiguous outcome is shown in Fig. 7. The results of this assay show five colors arising at the same threshold cycle (a, b, c, d, and e), one color of which (e) increases in intensity at twice the rate as the other four colors.

Example 7

[0045] The ambiguities inherent in results such as those shown in Fig. 7 can be resolved by repeating the assay with the same probes encoded by an alternative coding scheme. For example, the assay giving results shown in Fig. 8 is carried out on the same sample used to obtain the results shown in Fig. 7, except that the probes are encoded by the following alternative coding scheme (which I call the "second ternary color codebook"):

probe for species 0	cde
probe for species 1	bde
probe for species 2	bce
probe for species 3	bcd
probe for species 4	ade
probe for species 5	ace
probe for species 6	acd
probe for species 7	abe
probe for species 8	abd

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(continued)

probe for species 9 abc

[0046] Fig 8 shows the results that will be obtained from an assay on the same sample used in the experiment shown in Fig. 7, except that the probes are encoded according to the second ternary color codebook. A signal consisting of four different colors (a, b, c, and e) arises at the same threshold cycle. The slope of the curves for two of the colors (b and c) is twice as high as the slope of the curves for the other two colors (a and e). Since the slope of the curves for colors "b" and "c" are approximately twice as high as the slope of the curves for colors "a" and "e", colors "b" and "c" are present in the codes for both bacterial species that are present in the sample. The conclusion drawn from these results is that one of the probes that bound to amplified target sequences is encoded "abc" and the other probe that bound to amplified target sequences is encoded "bce". By comparing these results with the coding scheme in the second ternary color codebook (shown above), "bce" is seen to be the code for bacterial species "2" and "abc" is seen to be the code for bacterial species "9". Thus, the results shown in Fig. 8 resolve the ambiguity posed by the results shown in Fig. 7, unambiguously indicating that the sample contains species "2" and species "9" in approximately equal concentration.

[0047] Fig. 6b shows the results that should occur for all 45 different combinations of two different bacterial species in a sample at approximately the same concentration when the probes are encoded according to the second ternary color codebook. A comparison of the two-part table in Fig. 6a with the two-part table in Fig. 6b shows that all 45 possible combinations of two different bacterial species can be unambiguously identified by carrying out a second assay with alternatively coded probes when it is necessary to resolve ambiguities arising from the first assay.

[0048] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

Claims

1. A multiplexed homogeneous *in vitro* assay to screen a sample for the presence of at least six different nucleic acid targets comprising:

   a) contacting the sample with a set of at least six combinatorially coded fluorescent hybridization probes, each probe specific for a different nucleic acid target, wherein

      i) each probe in said set is labeled with a combinatorial code comprising at least two different emitter fluorophores selected from a panel of at least four spectrally differentiable fluorophores,

      ii) each target specific probe is subdivided into portions, each portion comprising a population of molecules, and wherein the number of portions is equal to the number of emitter fluorophores used to label each probe,

      iii) each portion is labeled with a different emitter fluorophore and wherein molecules of a portion are labeled with the same single emitter fluorophore,

      iv) said portions are mixed to create a single uniquely-labeled probe specific for one of said nucleic acid targets, and

      v) hybridization of each probe portion with its corresponding nucleic acid target leads to a detectable fluorescent signal change indicative of its labeling;

   b) stimulating the fluorophores present with the sample; and

   c) detecting changes in emissions signals from said emitter fluorophores without spatially segregating the nucleic acid targets from one another.

2. The assay according to claim 1 further comprising exponentially amplifying said nucleic acid targets, optionally wherein changes in emission signals are detected in real time during amplification.

3. The assay according to claim 2 wherein each probe comprises two, three or four differently labeled portions.

4. The assay according to any one of claims 1 to 3 wherein the relative amounts of the portions of each probe are adjusted relative to one another to provide balanced signal changes.

5. The assay according to any one of claims 1 to 4 wherein said set of probes additionally includes at least one probe that is ratio coded.

6. The assay according to any one of claims 1 to 5 wherein said combinatorially coded probes are quenched probes whose fluorescence is restored by hybridization to their targets, optionally wherein said combinatorially coded probes are molecular beacon probes having a non-fluorescent quencher on one arm and an emitter fluorophore on the other arm and preferably the fluorescence intensities of the portions of each probe are balanced relative to one another.

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7. The assay according to claim 6 further comprising exponentially amplifying said nucleic acid targets in the presence of said probes and detecting increases in fluorescence from emitter fluorophores in real time.

8. The assay according to claim 7 wherein each probe comprises two, three or four differently labeled portions from a panel of four to eight differentiable fluorophores, wherein the relative amounts of the portions of each probe are adjusted relative to one another to provide balanced fluorescence emissions, optionally wherein the relative amounts of all portions of all probes are adjusted relative to one another to provide balanced fluorescence emissions.

9. A kit of reagents for use in a multiplexed *in vitro* assay to screen a sample for the presence of at leastsix different nucleic acid targets comprising a set of at least six combinatorially coded fluorescent hybridization probes, each probe specific for a different nucleic acid target, wherein

   i) each probe in said set is labeled with a combinatorial code comprising at least two different emitter fluorophores selected from a panel of at least four spectrally differentiable fluorophores,
   ii) each target specific probe is subdivided into portions, each portion comprising a population of molecules, and wherein the number of portions is equal to the number of emitter fluorophores used to label each probe,
   iii) each portion is labeled with a different emitter fluorophore and wherein molecules of a portion are labeled with the same single emitter fluorophore,
   iv) said portions are mixed to create a single uniquely-labeled probe specific for one of said nucleic acid targets, and
   v) hybridization of each probe portion with its corresponding nucleic acid target leads to a detectable fluorescent signal change indicative of its labeling.

10. The kit according to claim 9 further comprising:

    (a) primers for exponential amplification of said nucleic acid targets;
    (b) amplification reagents for amplifying said nucleic acid targets utilizing said primers; or
    (c) at least one probe that is ratio coded.

11. The kit according to claim 9 or claim 10 wherein the combinatorially coded probes are quenched probes whose fluorescence is restored by hybridization to their targets, optionally wherein said probes are molecular beacon probes having a non-fluorescent quencher on one arm and an emitter fluorophore on the other arm.

12. The kit according to claim 11 wherein the relative amounts of the portions of each probe are adjusted relative to one another to provide balanced fluorescence emissions, optionally wherein the relative amounts of all portions of all probes are adjusted relative to one another to provide balanced fluorescence emissions.

Patentansprüche

1. Homogener Mehrfach-*in-vitro*-Assay zur Durchmusterung einer Probe auf die Anwesenheit von mindestens sechs Nukleinsäure-Targets, umfassend:

   a) Inkontaktbringen der Probe mit einem Satz von mindestens sechs kombinatorisch kodierten fluoreszierenden Hybridisierungssonden, wobei jede Sonde spezifisch ist für ein anderes Nukleinsäure-Target, wobei

      i) jede Sonde in dem Satz mit einem kombinatorischen Code markiert ist, der mindestens zwei verschiedene Emitterfluorophore umfasst, die ausgewählt sind aus einem Panel von mindestens vier spektral unterschiedlichen Fluorophoren,
      ii) jede targetspezifische Sonde in Abschnitte unterteilt ist, wobei jeder Abschnitt eine Population von Molekülen umfasst, und wobei die Anzahl der Abschnitte gleich der Anzahl von Emitterfluorophoren ist, die verwendet werden, um die einzelnen Sonden zu markieren,
      iii) jeder Abschnitt mit einem anderen Emitterfluorophor markiert ist, und wobei Moleküle eines Abschnitts mit dem gleichen, einzigen Emitterfluorophor markiert sind,
      iv) die Abschnitte gemischt sind, um eine einzige, eindeutig markierte Sonde zu erzeugen, die spezifisch ist für eines von den Nukleinsäure-Targets, und
      v) die Hybridisierung jedes Sondenabschnitts mit seinem entsprechenden Nukleinsäure-Target zu einer nachweisbaren Fluoreszenzsignaländerung führt, die für seine Markierung bezeichnend ist;

   b) Stimulieren der vorhandenen Fluorophore mit der Probe; und
   c) Nachweisen von Änderungen in Emissionssignalen von den Emitterfluorophoren, ohne die Nukleinsäure-Targets räumlich voneinander zu trennen.

2. Assay nach Anspruch 1, ferner das exponentielle

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Amplifizieren der Nukleinsäure-Targets umfassend, wobei Änderungen in Emissionssignalen während der Amplifizierung optional in Echtzeit erkannt werden.

3. Assay nach Anspruch 2, wobei jede Sonde zwei, drei oder vier unterschiedlich markierte Abschnitte umfasst.

4. Assay nach einem der Ansprüche 1 bis 3, wobei die relativen Mengen der Abschnitte jeder Sonde in Bezug aufeinander angepasst sind, um ausgewogene Signaländerungen zu ermöglichen.

5. Assay nach einem der Ansprüche 1 bis 4, wobei der Satz von Sonden außerdem mindestens eine Sonde umfasst, die verhältniscodiert ist.

6. Assay nach einem der Ansprüche 1 bis 5, wobei die kombinatorisch codierten Sonden gequenchte Sonden sind, deren Fluoreszenz durch Hybridisierung an ihre Targets wiederhergestellt wird, wobei die kombinatorisch codierten Sonden optional Molecular-Beacon-Sonden sind, die einen nicht-fluoreszierenden Quencher an einem Zweig und einen Emitterfluorophor am anderen Zweig aufweisen, und wobei die Fluoreszenzintensitäten der Abschnitte jeder Sonde in Bezug aufeinander vorzugsweise ausgeglichen sind.

7. Assay nach Anspruch 6, ferner das exponentielle Amplifizieren der Nukleinsäure-Targets in Anwesenheit der Sonden und das Nachweisen von Zunahmen der Fluoreszenz von Emitterfluorophoren in Echtzeit umfassend.

8. Assay nach Anspruch 7, wobei jede Sonde zwei, drei oder vier verschieden markierte Abschnitte aus einem Panel von vier bis acht unterscheidbaren Fluorophoren umfasst, wobei die relativen Mengen jeder Sonde in Bezug aufeinander angepasst sind, um ausgewogene Fluoreszenzemissionen zu ermöglichen, wobei die relativen Mengen aller Abschnitte aller Sonden in Bezug aufeinander optional angepasst sind, um ausgewogene Fluoreszenzemissionen zu ermöglichen.

9. Kit von Reagenzien zur Verwendung in einem Mehrfach-*in-vitro*-Assay zur Durchmusterung einer Probe auf die Anwesenheit von mindestens sechs verschiedenen Nukleinsäure-Targets, einen Satz von mindestens sechs kombinatorisch codierten fluoreszierenden Hybridisierungssonden umfassend, wobei jede Sonde spezifisch ist für ein anderes Nukleinsäure-Target, wobei

   i) jede Sonde in dem Satz mit einem kombinatorischen Code markiert ist, der mindestens zwei verschiedene Emitterfluorophore umfasst, die ausgewählt sind aus einem Panel von mindestens vier spektral unterschiedlichen Fluorophoren,
   ii) jede targetspezifische Sonde in Abschnitte unterteilt ist, wobei jeder Abschnitt eine Population von Molekülen umfasst, und wobei die Anzahl der Abschnitte gleich der Anzahl von Emitterfluorophoren ist, die verwendet werden, um die einzelnen Sonden zu markieren,
   iii) jeder Abschnitt mit einem anderen Emitterfluorophor markiert ist, und wobei Moleküle eines Abschnitts mit dem gleichen, einzigen Emitterfluorophor markiert sind,
   iv) die Abschnitte gemischt sind, um eine einzige, eindeutig markierte Sonde zu erzeugen, die spezifisch ist für eines von den Nukleinsäure-Targets, und
   v) die Hybridisierung jedes Sondenabschnitts mit seinem entsprechenden Nukleinsäure-Target zu einer nachweisbaren Fluoreszenzsignaländerung führt, die für seine Markierung bezeichnend ist;

10. Kit nach Anspruch 9, ferner umfassend:

    (a) Primer für eine exponentielle Amplifizierung der Nukleinsäure-Targets,
    (b) Amplifikationsreagenzien zum Amplifizieren der Nukleinsäure-Targets unter Verwendung der Primer; oder
    (c) mindestens eine Sonde, die verhältniscodiert ist.

11. Kit nach Anspruch 9 oder Anspruch 10, wobei die kombinatorisch codierten Sonden gequenchte Sonden sind, deren Fluoreszenz durch Hybridisierung an ihre Targets wiederhergestellt wird, wobei die Sonden optional Molecular-Beacon-Sonden sind, die einen nicht-fluoreszierenden Quencher an einem Zweig und einen Emitterfluorophor am anderen Zweig aufweisen.

12. Kit nach Anspruch 11, wobei die relativen Mengen der Abschnitte jeder Sonde in Bezug aufeinander angepasst sind, um ausgewogene Fluoreszenzemissionen zu ermöglichen, wobei die relativen Mengen aller Abschnitte aller Sonden in Bezug aufeinander optional angepasst sind, um ausgewogene Fluoreszenzemissionen zu ermöglichen.

Revendications

1. Essai *in vitro* homogène multiplexe servant à balayer un échantillon pour détecter la présence d'au moins six cibles d'acide nucléique différentes, comprenant :

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a) la mise en contact de l'échantillon avec au moins six échantillons d'hybridation fluorescent à codage combinatoire, chaque échantillon étant spécifique à une cible d'acide nucléique différente, dans lequel

i) chaque échantillon dudit ensemble est étiqueté avec un code combinatoire comprenant au moins deux fluorophores émetteurs différents sélectionnés dans un groupe d'au moins quatre fluorophores différenciables au niveau spectral,
ii) chaque échantillon spécifique à une cible est subdivisé en sections, chaque section comprenant un peuplement de molécules, et dans lequel le nombre de sections est égal au nombre de fluorophores émetteurs utilisés pour étiqueter chaque échantillon,
iii) chaque section est étiquetée avec un fluorophore émetteur différent et dans lequel les molécules d'une section sont étiquetées avec le même unique fluorophore émetteur,
iv) lesdites sections sont mélangées pour créer un seul échantillon à étiquetage unique spécifique à une desdites cibles d'acide nucléique ; et
v) l'hybridation de chaque section d'échantillon avec sa cible d'acide nucléique correspondante entraîne un changement de signal fluorescent détectable indiquant son étiquetage ;

b) la stimulation des fluorophores présents avec l'échantillon ; et
c) la détection de changements dans des signaux d'émissions desdits émetteurs fluorophores sans isoler spatialement les cibles d'acide nucléique les unes des autres.

2. Essai selon la revendication1, comprenant en outre l'amplification exponentielle desdites cibles d'acide nucléique, dans lequel, en option, les changements des signaux d'émissions sont détectés en temps réel pendant l'amplification.

3. Essai selon la revendication 2, dans lequel chaque échantillon comprend deux, trois ou quatre sections étiquetées différemment.

4. Essai selon l'une quelconque des revendications 1 à 3, dans lequel les quantités relatives des sections de chaque échantillon sont ajustées les unes par rapport aux autres pour permettre des changements de signaux équilibrés.

5. Essai selon l'une quelconque des revendications 1 à 4, dans lequel ledit ensemble d'échantillons inclut au moins un échantillon qui est codé par ratio.

6. Essai selon l'une quelconque des revendications 1 à 5, dans lequel lesdits échantillons à codage combinatoire sont des échantillons trempés dont la fluorescence est restaurée par hybridation vers leurs cibles, dans lequel, en option, lesdits échantillons à codage combinatoire sont des échantillons à balise moléculaire dotés d'une balise non fluorescente sur un bras et d'un fluorophore émetteur sur l'autre bras et les intensités de fluorescence des sections de chaque échantillon sont de préférence équilibrées entre elles.

7. Essai selon la revendication 6, comprenant en outre l'amplification exponentielle desdites cibles d'acide nucléique en présence desdits échantillons et la détection d'augmentations de fluorescence provenant des fluorophores émetteurs en temps réel.

8. Essai selon la revendication 7, dans lequel chaque échantillon comprend deux, trois ou quatre fluorophores étiquetés différemment sur un groupe de quatre à huit fluorophores différenciables, dans lequel les quantités relatives de chaque échantillon sont ajustées les unes par rapport aux autres, dans lequel, en option, les quantités relatives de toutes les sections de tous les échantillons sont ajustées les unes par rapport aux autres pour permettre des émissions à fluorescence équilibrée.

9. Ensemble de réactifs destinés à un usage dans un essai *in vitro* homogène multiplexe servant à balayer un échantillon pour détecter la présence d'au moins six cibles d'acide nucléique différentes, comprenant un ensemble d'au moins six échantillons d'hybridation fluorescents à codage combinatoire, chaque échantillon étant spécifique à une cible d'acide nucléique différente, dans lequel

i) chaque échantillon dudit ensemble est étiqueté avec un code combinatoire comprenant au moins deux fluorophores émetteurs sélectionnés dans un groupe d'au moins quatre fluorophores différenciables au niveau spectral,
ii) chaque échantillon spécifique à une cible est subdivisé en sections, chaque section comprenant un peuplement de molécules, et dans lequel le nombre de sections est égal au nombre de fluorophores émetteurs utilisés pour étiqueter chaque échantillon,
iii) chaque section est étiquetée avec un fluorophore émetteur différent et dans lequel les molécules d'une section sont étiquetées avec le même unique fluorophore émetteur,
iv) lesdites sections sont mélangées pour créer un seul échantillon à étiquetage unique spécifique à une desdites cibles d'acide nucléique ; et

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v) l'hybridation de chaque section d'échantillon avec sa cible d'acide nucléique correspondante entraîne un changement de signal fluorescent détectable indiquant son étiquetage.

10. Ensemble selon la revendication 9, comprenant en outre :

(a) des primaires pour l'amplification exponentielle desdites cibles d'acide nucléique ;
(b) des réactifs d'amplification pour amplifier lesdites cibles d'acide nucléique en utilisant lesdits primaires ;
(c) au moins un échantillon qui est codé par ratio.

11. Ensemble selon la revendication 9 ou la revendication 10, dans lequel les échantillons à codage combine sont des échantillons trempés dont la fluorescence est restaurée par hybridation vers leurs cibles, dans lequel, en option, lesdits échantillons à codage combiné sont des échantillons à balise moléculaire dotés d'une balise fluorescente sur un bras et d'un fluorophore émetteur sur l'autre bras.

12. Ensemble selon la revendication 11, dans lequel les quantités relatives des sections de chaque échantillon sont ajustées les unes par rapport aux autres pour permettre des émissions à fluorescence équilibrée, dans lequel, en option, les quantités relatives de toutes les sections de tous les échantillons sont ajustées les unes par rapport aux autres pour permettre des émissions à fluorescence équilibrée.

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TWO COLORS PER PROBE:
PANEL OF 4(a, b, c, d), 5(+e), 6(+f), 7(+g) or 8(+h) FLUOROPHORES

ab					}= 6	}		}		}		}		
ac	bc				}	}		}		}		}		
ad	bd	cd			}	} = 10		}		}		}		
						}		}		}		}		
ae	be	ce	de			}		} =15		}		}		
								}		}		}		
af	bf	cf	df	ef				}		} =21		}		
										}		}		
ag	bg	cg	dg	eg	fg					}		}= 28		
												}		
ah	bh	ch	dh	eh	fh	gh						}		

THREE COLORS PER PROBE:
PANEL OF 5(a, b, c, d, e), 6(+f), 7(+g) or 8(+h) FLUOROPHORES

abc					} =10	}		}		}		}				
abd					}	}		}		}		}				
abe					}	}		}		}		}				
acf	bcd				}	}		}		}		}				
ace	bce				}	}		}		}		}				
ade	bde	cde			}	}		}		}		}				
						}= 20		}		}		}				
abf						}		}		}		}				
acf	bcf					}		}		}		}				
adf	bdf	cdf				}		}		}		}				
aef	bef	cef	def			}		}		}		}				
										}= 35		}				
abg								}		}		}				
acg	bcg							}		}		}				
adg	bdg	cdg						}		}		}				
aeg	beg	ceg	deg					}		}		}				
afg	bfg	cfg	dfg	efg				}		}		}				
												}				
abh												}				
ach	bch											}= 56				
adh	bdh	cdh										}				
aeh	beh	ceh	deh									}				
afh	bfh	cfh	dfh	efh								}				
agh	bgh	cgh	dgh	egh	fgh							}				

FIGURE 1

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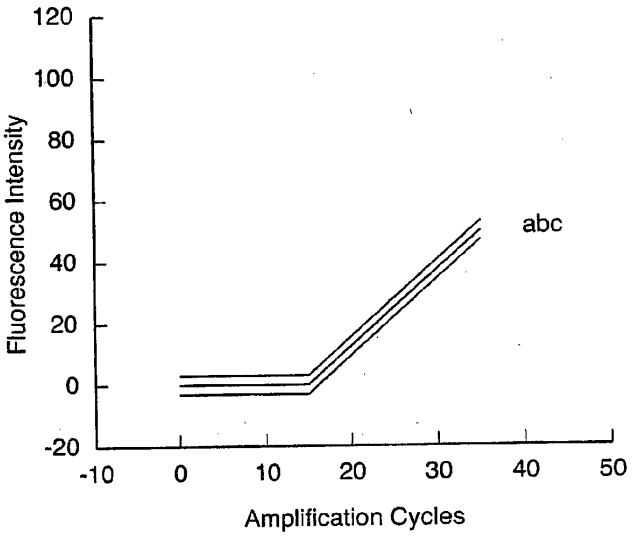


FIGURE 2

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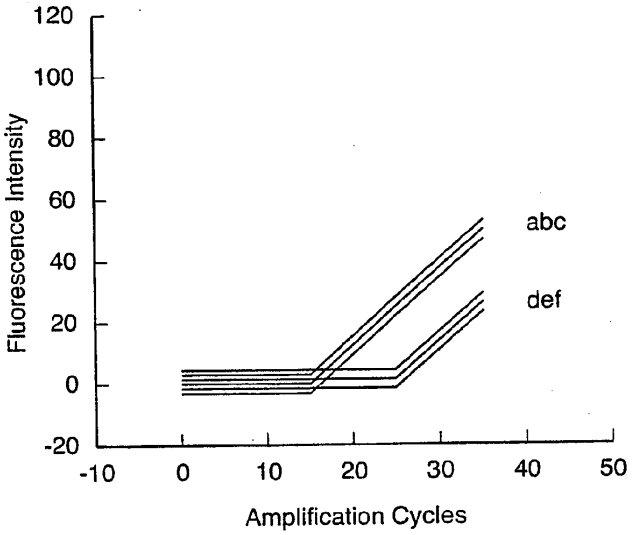


FIGURE 3

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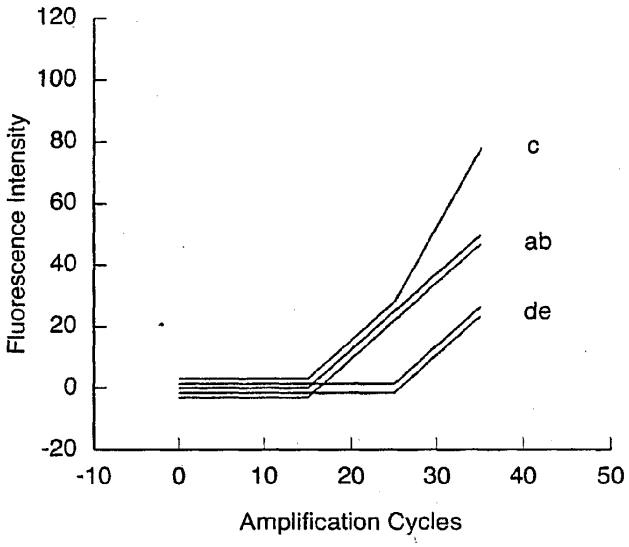


FIGURE 4

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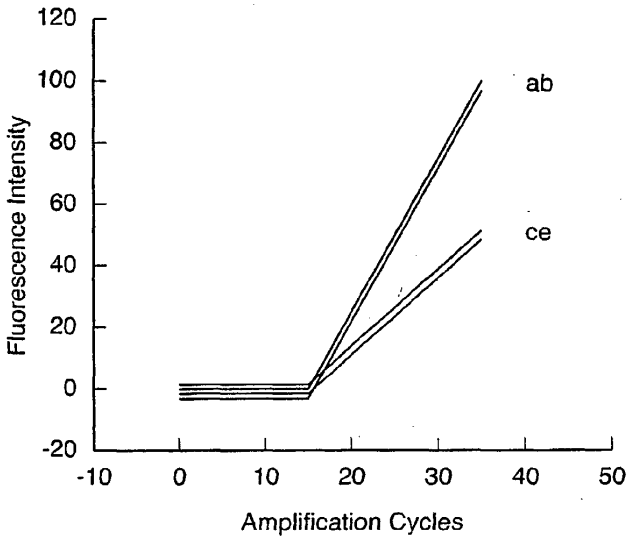


FIGURE 5

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FIRST TERNARY COLOR CODEBOOK

	0(abc)	1(abd)	2(abe)	3(acd)	4(ace)
0(abc)		0+1(abcd)	0+2(abce)	0+3(abcd)	0+4(abce)
1(abd)	0+1(abcd)		1+2(abde)	1+3(abcd)	1+4(abcde)
2(abe)	0+2(abce)	1+2(abde)		2+3(abcde)	2+4(abce)
3(acd)	0+3(abcd)	1+3(abcd)	2+3(abcde)		3+4(acde)
4(ace)	0+4(abce)	1+4(abcde)	2+4(abce)	3+4(acde)	
5(ade)	0+5(abcde)	1+5(abde)	2+5(abde)	3+5(acde)	4+5(acde)
6(bcd)	0+6(abcd)	1+6(abcd)	2+6(abcde)	3+6(abcd)	4+6(abcde)
7(bce)	0+7(abce)	1+7(abcde)	2+7(abce)	3+7(abcde)	4+7(abce)
8(bde)	0+8(abcde)	1+8(abde)	2+8(abde)	3+8(abcde)	4+8(abcde)
9(cde)	0+9(abcde)	1+9(abcde)	2+9(abcde)	3+9(acde)	4+9(acde)

	5(ade)	6(bcd)	7(bce)	8(bde)	9(cde)
0(abc)	0+5(abcde)	0+6(abcd)	0+7(abce)	0+8(abcde)	0+9(abcde)
1(abd)	1+5(abde)	1+6(abcd)	1+7(abcde)	1+8(abde)	1+9(abcde)
2(abe)	2+5(abde)	2+6(abcde)	2+7(abce)	2+8(abde)	2+9(abcde)
3(acd)	3+5(acde)	3+6(abcd)	3+7(abcde)	3+8(abcde)	3+9(acde)
4(ace)	4+5(acde)	4+6(abcde)	4+7(abce)	4+8(abcde)	4+9(acde)
5(ade)		5+6(abcde)	5+7(abcde)	5+8(abde)	5+9(acde)
6(bcd)	5+6(abcde)		6+7(bcde)	6+8(bcde)	6+9(bcde)
7(bce)	5+7(abcde)	6+7(bcde)		7+8(bcde)	7+9(bcde)
8(bde)	5+8(abde)	6+8(bcde)	7+8(bcde)		8+9(bcde)
9(cde)	5+9(acde)	6+9(bcde)	7+9(bcde)	8+9(bcde)	

FIGURE 6a

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SECOND TERNARY COLOR CODEBOOK

	9(abc)	8(abd)	7(abe)	6(acd)	5(ace)
9(abc)		8+9(abcd)	7+9(abce)	6+9(abcd)	5+9(abce)
8(abd)	8+9(abcd)		7+8(abde)	6+8(abcd)	5+8(abcde)
7(abe)	7+9(abce)	7+8(abde)		6+7(abcde)	5+7(abce)
6(acd)	6+9(abcd)	6+8(abcd)	6+7(abcde)		5+6(acde)
5(ace)	5+9(abce)	5+8(abcde)	5+7(abce)	5+6(acde)	
4(ade)	4+9(abcde)	4+8(abde)	4+7(abde)	4+6(acde)	4+5(acde)
3(bcd)	3+9(abcd)	3+8(abcd)	3+7(abcde)	3+6(abcd)	3+5(abcde)
2(bce)	2+9(abce)	2+8(abcde)	2+7(abce)	2+6(abcde)	2+5(abce)
1(bde)	1+9(abcde)	1+8(abde)	1+7(abde)	1+6(abcde)	1+5(abcde)
0(cde)	0+9(abcde)	0+8(abcde)	0+7(abcde)	0+6(acde)	0+5(acde)

	4(ade)	3(bcd)	2(bce)	1(bde)	0(cde)
9(abc)	4+9(abcde)	3+9(abcd)	2+9(abce)	1+9(abcde)	0+9(abcde)
8(abd)	4+8(abde)	3+8(abcd)	2+8(abcde)	1+8(abde)	0+8(abcde)
7(abe)	4+7(abde)	3+7(abcde)	2+7(abce)	1+7(abde)	0+7(abcde)
6(acd)	4+6(acde)	3+6(abcd)	2+6(abcde)	1+5(abcde)	0+6(acde)
5(ace)	4+5(acde)	3+5(abcde)	2+5(abce)	1+5(abcde)	0+5(acde)
4(ade)		3+4(abcde)	2+4(abcde)	1+4(abde)	0+4(acde)
3(bcd)	3+4(abcde)		2+3(bcde)	1+3(bcde)	0+3(bcde)
2(bce)	2+4(abcde)	2+3(bcde)		1+2(bcde)	0+2(bcde)
1(bde)	1+4(abde)	1+3(bcde)	1+2(bcde)		0+1(bcde)
0(cde)	0+4(acde)	0+3(bcde)	0+2(bcde)	0+1(bcde)	

FIGURE 6b

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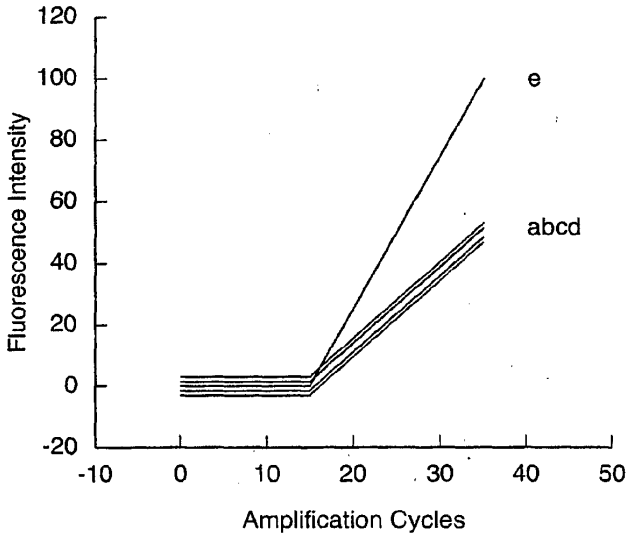


FIGURE 7

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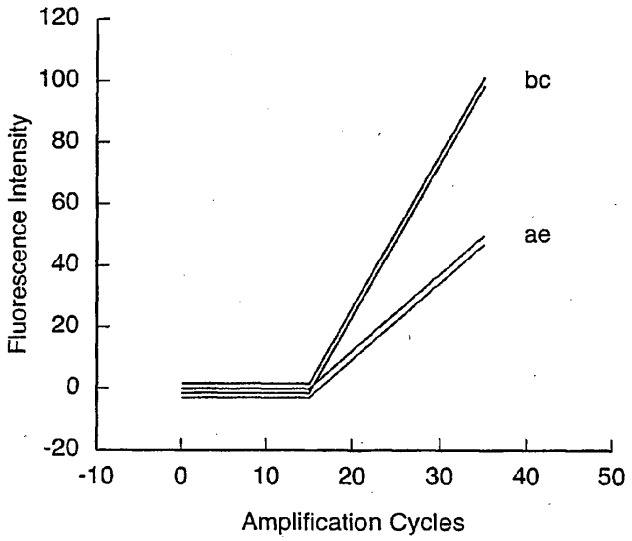


FIGURE 8

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REFERENCES CITED IN THE DESCRIPTION

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- **TYAGI, S. et al.** Wavelength-Shifting Molecular Beacons. *Nature Biotechnol.,* 2000, vol. 18, 1191-1196 **[0024]**
- **GAO et al.** Quantum-Dot Nanocrystals for Ultrasensitive Biological Labeling and Multicolor Optical Encoding. *J. Biomed. Opt.,* 2002, vol. 7, 532-537 **[0026]**
- **KANE, T.D. et al.** The Detection of Microbial DNA in Blood: a Sensitive Method for Diagnosing Bacteremia and/or Bacterial Translocation in Surgical Patients. *Ann. Surg.,* 1998, vol. 227, 1-9 **[0029]**

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- **LEE et al.** Seven-Color, Homogeneous Detection of Six PCR Products. *Biotechniques,* 1999, vol. 27, 342-349 **[0031]**

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Electronic Patent Application Fee Transmittal

Application Number:	14451876
Filing Date:	05-Aug-2014
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Filer:	Lee Alan Johnson/Frank Chen (LJ6)
Attorney Docket Number:	38075-716.301

Filed as Small Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	2806	1	90	90
		Total in USD ($)		**90**

Electronic Acknowledgement Receipt	
EFS ID:	29094705
Application Number:	14451876
International Application Number:	
Confirmation Number:	1065
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Customer Number:	21971
Filer:	Lee Alan Johnson/Frank Chen (LJ6)
Filer Authorized By:	Lee Alan Johnson
Attorney Docket Number:	38075-716.301
Receipt Date:	02-MAY-2017
Filing Date:	05-AUG-2014
Time Stamp:	16:18:53
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
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RAM confirmation Number	050317INTEFSW00002946232415
Deposit Account	
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The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		IDS38075-716-301-5-2-2017.pdf	293783 aa8f9c034054cb43d97809ab2ab88724cd934fff	yes	9
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Transmittal Letter		1	4	
	Information Disclosure Statement (IDS) Form (SB08)		5	9	
Warnings:					
Information:					
2	Foreign Reference	WO2004087950A2.pdf	2588693 97613b05e6a6fca530ad3abdc77b4a91af06c30c	no	62
Warnings:					
Information:					
3	Other Reference-Patent/App/Search documents	Z-NOA-6-2-2014-US13756760.pdf	439635 63df3d2975604d72aa18e740b9a5a4c8d49f3356	no	8
Warnings:					
Information:					
4	Foreign Reference	EP1629108B1.pdf	7339191 65d612d71f0810d657f808278394ab68f7b17de8	no	26
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30843 04309f7a9e63dc1f69511361e0294ac8099752ff	no	2
Warnings:					
Information:					

Total Files Size (in bytes):	10692145

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	KARTALOV; Emil et al.	Group Art Unit:	1631
Serial Number:	14/451,876	Examiner:	RUSSELL SCOTT NEGIN
Filing or 371 (c) Date:	2014-08-05	**CONFIRMATION NO:**	**1065**
Title:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

FILED ELECTRONICALLY ON: May 2, 2017

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

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A. ☐ *37 CFR § 1.97 (b).* This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☐ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☐ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☒ *37 CFR § 1.97(c).* Although this Information Disclosure Statement is being filed after the period specified in *37 CFR § 1.97(b),* above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☒ a fee of $90.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ *37 CFR § 1.97 (d).* Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of $180.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ *37 CFR §1.97 (e).* Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

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E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

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I. ☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of $90.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 23-2415 (Docket No.38075-716.301).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: May 2, 2017

By: /Lee A. Johnson/
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/451,876	08/05/2014	Emil Kartalov	38075-716.301	1065

21971 7590 06/06/2017

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1631	

NOTIFICATION DATE	DELIVERY MODE
06/06/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

Office Action Summary	Application No. 14/451,876	Applicant(s) KARTALOV ET AL.	
	Examiner Russell S. Negin	Art Unit 1631	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *3 November 2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *See Continuation Sheet* is/are pending in the application.

5a) Of the above claim(s) *See Continuation Sheet* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *49,51,53,55,56,75-81,83-88 and 90-92* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date *5/2/2017*.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

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Continuation of Disposition of Claims: Claims pending in the application are 1,2,4,5,9,13,17-19,22,27-29,34,35,37-39,42,43,45,47,49,51,53,55,56,75-88, and 90-92.
Continuation of Disposition of Claims: Claims withdrawn from consideration are 1,2,4,5,9,13,17-19,22,27-29,34,35,37-39,42,43,45,47, and 82.

DETAILED ACTION

Comments

The present application is being examined under the pre-AIA first to invent provisions. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

Claims 1-2, 4-5, 9, 13, 17-19, 22, 27-29, 34-35, 37-39, 42-43, 45, 47, and 82 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected Group or Species, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on 3 November 2016.

Claims 1-2, 4-5, 9, 13, 17-19, 22, 27-29, 34-35, 37-39, 42-43, 45, 47, 49, 51, 53, 55-56, 75-88, and 90-92 are pending in the instant application.

Claims 49, 51, 53, 55-56, 75-81, 83-88, and 90-92 are examined in the instant Office action.

Information Disclosure Statements

The IDS filed on 5/2/2017 been considered.

Withdrawn Rejections

The rejection of claim 89 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention is withdrawn in view of cancellation of claim 89.

The prior art rejections are withdrawn in view of arguments on pages 8-13 of the Remarks.

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

The following rejection is reiterated:

Claims 49, 51, 53, 55-56, 75-81, 83-88, and 90-92 are rejected under 35 U.S.C. 101 because the claimed invention is directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more. According to Step 1 of the 2014 Interim Subject Matter Eligibility Guidelines, claim(s) 49, 51, 53, 55-56, 75-81, 83-88, and 90-92 is/are directed to methods. According to Step 2A of the 2014 Interim Subject Matter Eligibility Guidelines, claim(s) 49, 51, 53, 55-56, 75-81, 83-88, and 90-92 is/are directed to the abstract idea of encoding signals in a manner that reduces or eliminates degeneracy. The signal processing is a manner of mathematical manipulation analogous to the Arrhenius equation of *Diamond v. Diehr*, 450 U.S. 175 (1981), which was also found to be abstract. The claim(s) does/do not

include additional elements that are sufficient to amount to significantly more than the judicial exception (i.e. Step 2B of the Guidelines) because providing samples, performing assays, and determine presence/absence patterns of analytes is routine and conventional in the prior art. This rejection has been made in view of the U.S. Supreme Court Decision in *Alice Corporation Pty. Ltd. V. CLS Bank International, et al*.

Response to arguments:

Applicant's arguments filed 2 May 2017 have been fully considered but they are not persuasive.

On page 15 of the Remarks, applicant highlights the portions of the claim that are not part of any judicial exception. However, while there are five limitations highlighted, these limitations are routine and conventional in the prior art. Consequently, these limitations do not constitute significantly more than the abstract idea.

On pages 16-18 of the Remarks, applicant attempts to compare the fact pattern of the instant set of claims to *BASCOM*. The claims in *BASCOM* were found to be patent eligible because the judicial exceptions could only be performed within a computer. On page 15 of the Remarks, applicant highlights limitations of the claim that require the judicial exception be performed in combination with empirical wet-lab steps.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of pre-AIA 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

The following rejection is necessitated by the IDS of 5/2/2017:

Claim(s) 49, 51, 53, 55-56, 75-78, 80-81, 84-88, and 90-92 is/are rejected under pre-AIA 35 U.S.C. 102(e) as being anticipated by Lin et al. [US PGPUB 2013/0261019 A1; on IDS of 5/2/2017].

Claim 49 is drawn to a method of detecting the presence or absence of each analyte of a plurality of analytes. The method comprises encoding each of the analytes as a first value of a signal, thereby generating a coding scheme, wherein each of the analytes is represented in the coding scheme by the first value, wherein the encoding is performed in a manner that reduces or eliminates degeneracy. The method comprises providing a sample comprising at least one of said analytes. The method comprises contacting the sample with analyte-specific reagents that generate the first value, as represented in the coding scheme, when each of the analytes is present. The method comprises cumulatively measuring the first values of the signal within the sample, thereby providing a cumulative measurement. The method comprises determining whether each of the analytes is present or absent based on the cumulative measurement and the coding scheme. The method non-degenerately detects the presence or absence of at least seven analytes in a single sample volume, in any

combination of presence or absence. The method does not allow immobilization of analytes, physical separation of the analytes, or mass spectrometry.

Claim 55 is drawn to similar subject matter as claim 49, the additional limitation that each of the analytes corresponds to both a first value and a second value.

Lin et al. studies nucleic acid nanostructure barcode probes [title]. Paragraph 83 of Lin et al. teaches creation of a DNA barcode. Each oligonucleotide was attached to three values/fluorophores from the group consisting of rhodamine-green, Cy3, and Cy5. Figure 5D of Lin et al. illustrates all possible of the 27 nondegenerate combinations of barcodes. Claim 1 of Lin et al. teaches providing the analyte and contacting the analyte with the barcode probe. The histogram of Figure 5D of Lin et al. illustrates the cumulative measurement of the presence/absence patterns of each of the 27 non-degenerate barcode probes. Each of the 27 non-zero bars indicates presence of the 27 barcode probes. The methods used in Lin et al. do not involve immobilization of analytes, physical separation of analytes, or mass spectrometry.

With regard to claim 49, 51, 56, and 75-78; each of the dyes of rhodamine-green, Cy3, and Cy5 of paragraph 83 of Lin et al. correspond to the wavelength ranges for blue, green, and red. The three colors of the barcode correspond to three components of the signal of the barcode.

With regard to claim 53, Figure 5B of Lin et al. illustrates that the correct measurements is greater than the sum of the incorrect measurements.

With regard to claims 80-81 and 86, the oligonucleotides to which the dyes are attached to in paragraph 83 of Lin et al. are interpreted to function as hybridization probes. Paragraph 98 of Lin et al. teaches hybridization of the barcode probes to analytes to detect target polynucleotide analytes.

With regard to claims 84-85, Figure 5D of Lin et al. illustrates a diagram comprising all the possible combinations of barcodes.

With regard to claims 87-88, paragraph 102 of Lin et al. teaches a computer network to transmit information to a user such as a health care professional.

With regard to claim 90, Figure 5D of Lin et al. illustrates non-degenerate, legitimate results. No results are eliminated because the data in Figure 5D of Lin et al. are non-degenerate.

With regard to claims 91-92, Lin et al. does not teach immobilization of the barcode labeled probes.

Response to arguments:

Applicant's arguments with respect to the instantly rejected claims have been considered but are moot because the arguments do not apply to any of the references being used in the current rejection.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

The following rejection is necessitated by the IDS of 5/2/2017:

35 U.S.C. 103 Rejection #1:

Claim 79 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Lin et al. as applied to claims 49, 51, 53, 55-56, 75-78, 80-81, 84-88, and 90-92 above in further view of Nie et al. [US PGPUB 2003/0148544 A1; on IDS].

Claim 79 is further limiting wherein an electromagnetic signal is generated.

Lin et al. teaches optical encoding of analytes in microbeads as a result of intensities and wavelengths of optical signals emitted from the analytes, as discussed above.

Lin et al. does not teach electromagnetic signals.

The document of Nie et al. studies methods of preparing multicolor quantum dot tagged beads [title]. The cover figure of Nie et al. teaches that different color quantum dots within microbeads. Each type of quantum dot emits light at a different wavelength. The histograms in the cover figure of Nie et al. illustrates how the quantum dot composition within each microbead encodes a non-degenerate spectral signature in the form of intensity versus wavelength histogram. It is interpreted that the photons emitted from the quantum dots in the microbeads in the cover figure of Nie et al. to result in colored light comprises electromagnetic signaling.

It would have been obvious for someone of ordinary skill in the art at the time of the invention to modify the optical encoding of Lie et al. by use of the electromagnetic encoding of Nie et al. because it is obvious to combine known elements in the prior art to yield a predictable result. In this instance, optical encoding is an alternative to electromagnetic encoding. There would have been a reasonable expectation of success in combining Nie et al. and Craig et al. because both studies are analogously applicable to using presence/absence patterns as signatures for analyte combinations.

Response to arguments:

Applicant's arguments filed 2 May 2017 have been fully considered but they are not persuasive.

While Nie et al. is reiterated from the previous Office action, there are no arguments with regard to Nie et al. in the Remarks specific to claim 79.

The following rejection is necessitated by the IDS of 5/2/2017:

35 U.S.C. 103 Rejection #2:

Claim 83 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Lin et al. as applied to claims 49, 51, 53, 55-56, 75-78, 80-81, 84-88, and 90-92 above in further view of Orpana et al. [WO 2010/128206 A1].

Claim 83 is further limiting wherein the signal is generated by PCR.

Lin et al. teaches using presence/absence patterns of biomolecules in coding schemes, as discussed above.

Lin et al. does not teach PCR.

Orpana et al. studies a method, instrument, and computer program product for quantification of PCR products [title].

It would have been obvious for someone of ordinary skill in the art at the time of the invention to modify the optical encoding of Lin et al. by use of the computerized PCR of Orpana et al. wherein the motivation would have been that computer automate the analysis and PCR facilitates understanding of the experiment by amplifying the results of the experiment [title and abstract of Orpana et al.].

Response to arguments:

Applicant's arguments filed 2 May 2017 have been fully considered but they are not persuasive.

While Orpana et al. is reiterated from the previous Office action, there are no arguments with regard to Orpana et al. in the Remarks specific to claim 83.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be

commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

The following rejection is necessitated by the IDS of 5/2/2017:

Claims 49, 51, 53, 55, and 56 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 20-24, respectively, of U.S. Patent No. 8,838,394 B2 [on IDS of 5/2/2017]. Although the claims at issue are not identical, they are not patentably distinct from each other because both sets of claims are drawn to encoding analytes in a manner that eliminated degeneracy and determining

presence/absence patterns using the barcode probes. The claims of '394 recite limitations in addition to the limitations of the instant claims and thus, anticipate the instant claims.

Response to arguments:

This rejection is newly applied.

E-mail Communications Authorization

Per updated USPTO Internet usage policies, Applicant and/or applicant's representative is encouraged to authorize the USPTO examiner to discuss any subject matter concerning the above application via Internet e-mail communications. See MPEP 502.03. To approve such communications, Applicant must provide written authorization for e-mail communication by submitting the following statement via EFS-Web (using PTO/SB/439) or Central Fax (571-273-8300):

> Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned and practitioners in accordance with 37 CFR 1.33 and 37 CFR 1.34 concerning any subject matter of this application by video conferencing, instant messaging, or electronic mail. I understand that a copy of these communications will be made of record in the application file.

Written authorizations submitted to the Examiner via e-mail are **NOT** proper. Written authorizations must be submitted **via EFS-Web** (using PTO/SB/439) **or Central Fax** (571-273-8300). A paper copy of e-mail correspondence will be placed in the patent application when appropriate. E-mails from the USPTO are for the sole use of the intended recipient, and may contain information subject to the confidentiality requirement set forth in 35 USC § 122. See also MPEP 502.03.

Conclusion

No claim is allowed.

Applicant's submission of an information disclosure statement under 37 CFR 1.97(c) with the fee set forth in 37 CFR 1.17(p) on 2 May 2017 prompted the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 609.04(b). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 10:30 am to 5 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Peter Paras, Supervisory Patent Examiner, can be reached at (571) 272-4517.

Information regarding the status of the application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only.

/Russell S. Negin/
Primary Examiner, Art Unit 1631
31 May 2017

/RSN/ 5/30/2017

Search Strategy

Databases: BIOSIS Previews®, Embase®, Gale Group Health Periodicals Database, Gale Group PharmaBiomed Business Journals, Gale Group PROMT®, MEDLINE®

Set#	Searched for	Results
S10	encode and cumulative and scheme and degeneracy	2°
S9	encode and cumulative and scheme	141°
S8	encode and cumulative and analytes	3°
S7	encode and cumulative and signal and analytes	2°
S6	encode and cumulative and signal	568°
S5	encode and cumulative	920°
S4	encode and degeneracy	120°
S3	encode AND decode and degeneracy	4°
S2	coding AND encode AND decode and degeneracy	1°
S1	coding AND encode AND decode	314°

° Duplicates are removed from the search and from the result count.

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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 5

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	RUSSELL SCOTT NEGIN
Attorney Docket Number	38075-716.301

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
/RSN/	001	US-7930106	04-19-2011	CARRICK; James M.	
/RSN/	002	US-8039215	10-18-2011	HIGUCHI; Russell et al.	
/RSN/	003	US-8426132	04-23-2013	LI; Bin et al.	
/RSN/	004	US-8455184	06-04-2013	ATCHLEY; Daniel H. et al.	
/RSN/	005	US-8492094	07-23-2013	DIMITROV; Krassen et al.	
/RSN/	006	US-8519115	08-27-2013	WEBSTER; Philippa J. et al.	
/RSN/	007	US-8614061	12-24-2013	BRABETZ; Werner et al.	
/RSN/	008	US-8771955	07-08-2014	REED; Michael W. et al.	
/RSN/	009	US-8838394	09-16-2014	KARTALOV; Emil et al.	
/RSN/	010	US-8877464	11-04-2014	BABIEL; Reiner et al.	
/RSN/	011	US-8962250	02-24-2015	STANLEY; Keith	
/RSN/	012	US-9133506	09-15-2015	KATZIR; Nir et al.	
/RSN/	013	US-9222128	12-29-2015	SAXONOV; Serge et al.	
/RSN/	014	US-9260761	02-16-2016	TYAGI; Sanjay et al.	
/RSN/	015	US-9422593	08-23-2016	ROTHMANN; Thomas et al.	
/RSN/	016	US-9447457	09-20-2016	CHUN; Jong Yoon et al.	
/RSN/	017	US-20130017971	01-17-2013	GEISS; Gary K. et al.	
/RSN/	018	US-20130261019	10-03-2013	LIN; Chenxiang et al.	
/RSN/	019	US-20140038195	02-06-2014	MALIK; Imran R. et al.	
/RSN/	020	US-20150140554	05-21-2015	SNYDER; Richard O. et al.	

Examiner Signature	/RUSSELL S NEGIN/	Date Considered	05/30/2017

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 5

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	RUSSELL SCOTT NEGIN
Attorney Docket Number	38075-716.301

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
/RSN/	001	EP-1629108-B1	12-31-2014	PHRI PROPERTIES INC [US]		☐
/RSN/	002	WO-2004087950-A2	10-14-2004	ROCHE DIAGNOSTICS GMBH [DE], et al.		☐

Examiner Signature	/RUSSELL S NEGIN/	Date Considered	05/30/2017

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 3 of 5

Complete if Known	
Application Number	14451876
Filing Date	08-05-2014
First Named Inventor	KARTALOV; Emil
Art Unit	1631
Examiner Name	RUSSELL SCOTT NEGIN
Attorney Docket Number	38075-716.301

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
/RSN/	001	Notice of allowance dated 06/02/2014 for US Application No. 13/756,760.	☐

Examiner Signature	/RUSSELL S NEGIN/	Date Considered	05/30/2017

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	14451876	KARTALOV ET AL.
	Examiner	Art Unit
	RUSSELL S NEGIN	1631

CPC- SEARCHED		
Symbol	Date	Examiner
G06F 19/12 with modified terms	5/30/2017	RSN
G06F 19/18 with modified terms	5/30/2017	RSN
G06F 19/34 with modified terms	5/30/2017	RSN
G06F 19/3437 with modified terms	5/30/2017	RSN

CPC COMBINATION SETS - SEARCHED		
Symbol	Date	Examiner

US CLASSIFICATION SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
see attached search history printouts	5/30/2017	RSN
EAST prior art search	5/30/2017	RSN
EAST inventor search on all inventors	5/30/2017	RSN
EAST assignee search	5/30/2017	RSN
PALM inventor search on all inventors	5/30/2017	RSN

INTERFERENCE SEARCH			
US Class/ CPC Symbol	US Subclass / CPC Group	Date	Examiner

	/RUSSELL S NEGIN/ Primary Examiner.Art Unit 1631

/RSN/ 5/30/2017

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
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L2	79	kartalov.in. near emil.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:05
L3	116	rajagopal.in. near aditya.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:05
L4	732	scherer.in. near axel.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:05
L5	4664	"california institute of technology".as.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:06
L6	5016	g06f19/12.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:06
L7	12542	g06f19/18.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:06
L8	2272	g06f19/34.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:06

L9	11715	g06f19/3437.cpc.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:06
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L16	3	l14 and l15	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:07
L17	195032	"bar code"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:07
L18	755	l11 same l17	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:07

L19	301656	cumulative	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:08
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L22	80	l20 and l21	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:08
L23	10386939	components	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:08
L24	69	l22 and l23	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:08
L25	1039626	coding	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:08
L26	8	l24 and l25	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:09
L27	60411	degeneracy	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2017/05/30 13:09
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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 2361

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IPR2024-01177
California Institute of Technology - Ex. 2003 Page 2362

Electronically Filed On: August 11, 2017

PATENT
Attorney Docket No.: 38075-716.301

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application	)	
Inventors: Emil KARTALOV, *et al.*	)	Confirmation No.: 1065
Application No.: 14/451,876	)	Art Unit: 1631
Filed: August 5, 2014	)	Examiner: Russell Scott NEGIN
Title: **SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS**	)	Customer No. 021971

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO FINAL OFFICE ACTION AND REQUEST FOR CONSIDERATION UNDER AFCP 2.0

Dear Sir:

This paper responds to the Final Office Action mailed June 6, 2017, setting an initial due date of September 6, 2017. Applicants have replied within the shortened statutory period for reply. Accordingly, this response is timely filed, and no fees are believed to be due.

Amendments to the Claims begins on page **2** of this paper.
Remarks begin on page **8** of this paper.
Conclusion begin on page **21** of this paper.

IPR2024-01177
California Institute of Technology - Ex. 2003 Page 2363

CERTIFICATION AND REQUEST FOR CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0

Practitioner Docket No.:	Application No.:	Filing Date:
38075-716.301	14/451,876	August 5, 2014
First Named Inventor:	**Title:**	
EMIL P. KARTALOV	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS CONSIDERATION UNDER THE AFTER FINAL CONSIDERATION PILOT PROGRAM 2.0 (AFCP 2.0) OF THE ACCOMPANYING RESPONSE UNDER 37 CFR 1.116.

1. The above-identified application is (i) an original utility, plant, or design nonprovisional application filed under 35 U.S.C. 111(a) [a continuing application (*e.g.*, a continuation or divisional application) is filed under 35 U.S.C. 111(a) and is eligible under (i)], or (ii) an international application that has entered the national stage in compliance with 35 U.S.C. 371(c).
2. The above-identified application contains an outstanding final rejection.
3. Submitted herewith is a response under 37 CFR 1.116 to the outstanding final rejection. The response includes an amendment to at least one independent claim, and the amendment does not broaden the scope of the independent claim in any aspect.
4. This certification and request for consideration under AFCP 2.0 is the only AFCP 2.0 certification and request filed in response to the outstanding final rejection.
5. Applicant is willing and available to participate in any interview requested by the examiner concerning the present response.
6. This certification and request is being filed electronically using the Office's electronic filing system (EFS-Web).
7. Any fees that would be necessary consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, extension of time fees, are being concurrently filed herewith. [There is no additional fee required to request consideration under AFCP 2.0.]
8. By filing this certification and request, applicant acknowledges the following:

- Reissue applications and reexamination proceedings are not eligible to participate in AFCP 2.0.
- The examiner will verify that the AFCP 2.0 submission is compliant, *i.e.*, that the requirements of the program have been met (see items 1 to 7 above). For compliant submissions:
  - The examiner will review the response under 37 CFR 1.116 to determine if additional search and/or consideration (i) is necessitated by the amendment and (ii) could be completed within the time allotted under AFCP 2.0. If additional search and/or consideration is required but cannot be completed within the allotted time, the examiner will process the submission consistent with current practice concerning responses after final rejection under 37 CFR 1.116, *e.g.*, by mailing an advisory action.
  - If the examiner determines that the amendment does not necessitate additional search and/or consideration, or if the examiner determines that additional search and/or consideration is required and could be completed within the allotted time, then the examiner will consider whether the amendment places the application in condition for allowance (after completing the additional search and/or consideration, if required). If the examiner determines that the amendment does not place the application in condition for allowance, then the examiner will contact the applicant and request an interview.
    - The interview will be conducted by the examiner, and if the examiner does not have negotiation authority, a primary examiner and/or supervisory patent examiner will also participate.
    - If the applicant declines the interview, or if the interview cannot be scheduled within ten (10) calendar days from the date that the examiner first contacts the applicant, then the examiner will proceed consistent with current practice concerning responses after final rejection under 37 CFR 1.116.

Signature	Date
/Lee A. Johnson/	August 11, 2017
Name (Print/Typed) Lee A. Johnson	**Practitioner Registration No.** 75,671

***Note**: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required, see below*.*

☒ * Total of 1 forms are submitted.

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PTO/AIA/26 (04-13)
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TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT	Docket Number (Optional) 38075-716.301

In re Application of: EMIL P. KARTALOV, *et al.*

Application No.: 14/451,876

Filed: AUGUST 5, 2014

For: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

The applicant, CALIFORNIA INSTITUTE OF TECHNOLOGY, owner of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of **prior patent** No. 8,838,394 as the term of said **prior patent** is presently shortened by any terminal disclaimer. The applicant hereby agrees that any patent so granted on the instant application shall be enforceable only for any during such period that it and the **prior patent** are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the applicant does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the **prior patent**, "as the term of said **prior patent** is presently shortened by any terminal disclaimer," in the event that said **prior patent** later:

expires for failure to pay a maintenance fee;
is held unenforceable;
is found invalid by a court of competent jurisdiction;
is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
has all claims canceled by a reexamination certificate;
is reissued; or
is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

Check either box 1 or 2 below, if appropriate.

1. ☐ The undersigned is the applicant. If the applicant is an assignee, the undersigned is authorized to act on behalf of the assignee.

I hereby acknowledge that any willful false statements made are punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

2. ☒ The undersigned is an attorney or agent of record. Reg. No. 75,671

/Lee A. Johnson/ — Signature
August 11, 2017 — Date

Lee A. Johnson — Typed or printed name

Attorney of Record — Title
650.493.9300 — Telephone Number

☒ Terminal disclaimer fee under 37 CFR 1.20(d) included.

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This collection of information is required by 37 CFR 1.321. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Electronic Patent Application Fee Transmittal					
Application Number:	14451876				
Filing Date:	05-Aug-2014				
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS				
First Named Inventor/Applicant Name:	Emil Kartalov				
Filer:	Vernon A. Norviel/Katherine Lopez				
Attorney Docket Number:	38075-716.301				
Filed as Small Entity					
Filing Fees for Utility under 35 USC 111(a)					
Description		**Fee Code**	**Quantity**	**Amount**	**Sub-Total in USD($)**
Basic Filing:					
Pages:					
Claims:					
Miscellaneous-Filing:					
Petition:					
Patent-Appeals-and-Interference:					
Post-Allowance-and-Post-Issuance:					
Extension-of-Time:					

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
STATUTORY OR TERMINAL DISCLAIMER	2814	1	160	160
		Total in USD ($)		**160**

Electronic Acknowledgement Receipt	
EFS ID:	30059745
Application Number:	14451876
International Application Number:	
Confirmation Number:	1065
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil Kartalov
Customer Number:	21971
Filer:	Vernon A. Norviel/Katherine Lopez
Filer Authorized By:	Vernon A. Norviel
Attorney Docket Number:	38075-716.301
Receipt Date:	11-AUG-2017
Filing Date:	05-AUG-2014
Time Stamp:	16:58:11
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		CIT_38075_716_301_Resp_OA.pdf	639795 e573b8ebb52b776627dfaa1f66eb5c2a9f9df2d8	yes	21

	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Applicant Arguments/Remarks Made in an Amendment		8	21	
	Claims		2	7	
	Response After Final Action		1	1	
Warnings:					
Information:					
2	After Final Consideration Program Request	CIT_38075_716_301_Signed_AFCP.pdf	196550 d7ce943428e81cd505cf203324359d02969fcad3	no	1
Warnings:					
Information:					
3	Terminal Disclaimer Filed	CIT_38075_716_301_Signed_TD.pdf	106365 523a05970b3bc531e6d35827c57ffc2b07003448	no	1
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30728 42a7f283c3e9c1ebcfc7f21a1f349c0f95b032bc	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	973438		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

REMARKS

I. Status of the Claims

The following remarks are in response to the Examiner's Office Action mailed on June 6, 2017. Claims 49, 51, 53, 55-56, 75-81, 83-88, and 90-92 are pending. Claims 3, 6-8, 10-12, 14-16, 20-21, 23-26, 30-33, 36, 40-41, 44, 46, 48, 50-52, 54, 57-75, and 89 are canceled without prejudice. Claims 1-2, 4-5, 9, 13, 17-19, 22, 27-29, 34-35, 37-39, 42-43, 45, 47, and 82 are withdrawn. Claims 49, 55, and 76-78 are amended herein. New claims 93-95 are added herein. Support for the amended and new claims is found in the original claims and throughout the specification. No new matter is introduced by any amendment made herein.

Upon entry of the proposed claims, claims 49, 53, 55, 56, 76-81, 83-88, and 90-95 will be under examination. Favorable consideration of these claims is respectfully requested in light of the present amendments and the following remarks.

II. Claim Rejections Under 35 U.S.C. §101

The Office maintained the rejection of claims 49, 51, 53, 55, 56, 75-81, 83-88, and 90-92 under 35 U.S.C. 101, asserting that these claims were directed to a judicial exception (i.e., a law of nature, a natural phenomenon, or an abstract idea) without significantly more. *See* Office Action at pp. 3-4. This rejection is respectfully traversed in view of the present amendments, for at least the following reasons.

As an initial point, Applicant sumbits that instant claim 49 is directed to a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

a) encoding each of said analytes as at least one first value in a first component of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by one of said first values, wherein said first value is an intensity or range of intensities, and wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

b) providing a sample comprising, or potentially comprising, at least one of said analytes;

c) contacting said sample with analyte-specific reagents that generate said first values, as

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represented in said coding scheme, when each of said analytes is present;

d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and

e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme;

wherein said method non-degenerately detects the presence or absence of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization of said analytes, physical separation of said analytes, or mass spectrometry.

Instant claim 55 is directed to a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said first value is an intensity or range of intensities and said second value is a wavelength or range of wavelengths, and wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

b) providing a sample comprising, or potentially comprising, at least one of said analytes;

c) contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present;

d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and

e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme;

wherein said method, without immobilization of said analytes, physical separation of said analytes, or mass spectrometry, non-degenerately detects the presence or absence of each of at least

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six said analytes in a single sample volume, in any combination of presence or absence, when each of said reagents generates only one second value.

Applicant respectfully submits that the Office has inappropriately oversimplified the rejected independent claim to its "gist" or core principles by asserting that the claim is directed to "the abstract idea of encoding signals in a manner that reduces or eliminates degeneracy." *Id.* at page 3.

Applicant's position regarding the inappropriate oversimplification engaged by the Office is of record. To reiterate, the Federal Circuit, in *McRO, Inc. dba Planet Blue v. Bandai Namco Games America Inc.*, 120 USPQ2d 1091 (Fed. Cir. 2016), cautioned against distilling the claims of an invention into its "gist" or "core principles." As noted in the response filed on May 2, 2017, the *McRO* court cautioned that courts "must be careful to avoid oversimplifying the claims" by looking at them generally and failing to account for the specific requirements of the claims." *Id.* at page 2 (emphasis added). The teaching of *McRO* is further emphasized in a memorandum issued on Nov. 2, 2016 by Robert W. Bahr, the Deputy Commissioner for Patent Examination Policy, regarding "Recent Subject Matter Eligibility Decisions" (hereinafter "Nov. 2016 Eligibility Memo"). Under the section titled "Notable Points from McRO" at page 2, the Nov. 2016 Eligibility Memo stated that "[e]xaminers should consider the claim as a whole under Step 2A of the USPTO's SME guidance, and should not overgeneralize the claim or simplify it into its 'gist' or core principles, when identifying a concept as a judicial exception."

A. The Steps of the Instant Invention Provide Specific and Meaningful Limitations

In *McRO*, the court examined claims to a method of "automatically animating lip synchronization and facial expression of three-dimensional characters." *McRO*, 837 F.3d at 1307. Specifically, the claimed method describes a set of rules designed to automate a 3-D animator's tasks. *Id.* The claims recite:

> A method for automatically animating lip synchronization and facial expression of three-dimensional characters comprising:
>
> obtaining a first set of rules that define output morph weight set stream as a function of phoneme sequence and time of said phoneme sequence;

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> obtaining a timed data file of phonemes having a plurality of sub-sequences;
>
> generating an intermediate stream of output morph weight sets and a plurality of transition parameters between two adjacent morph weight sets by evaluating said plurality of sub-sequences against said first set of rules;
>
> generating a final stream of output morph weight sets at a desired frame rate from said intermediate stream of output morph weight sets and said plurality of transition parameters; and
>
> applying said final stream of output morph weight sets to a sequence of animated characters to produce lip synchronization and facial expression control of said animated characters.

Id. at 1307-08.

The district court had found these claims invalid under §101. *Id.* at 1309. In its analysis, the district court determined that the claims were "too broadly preemptive to satisfy § 101 [ ] because the claims were not limited to specific rules, but rather 'purport to cover all such rules,' the claims merely call for application of the abstract idea of using rules." *Id.* The Federal Circuit disagreed and held that:

> Here, the claims are limited to rules with specific characteristics. As the district court recognized during claim construction, "the claims themselves set out meaningful requirements for the first set of rules: they 'define[] a morph weight set stream as a function of phoneme sequence and times associated with said phoneme sequence.'" J.A. 4171 (Dist. Ct. Claim Construction Op. 16) (quoting '567 patent, cl. 1). They further require "applying said first set of rules to each sub-sequence . . . of timed phonemes." *Id.* Whether at step one or step two of the *Alice* test, in determining the patentability of a method, a court must look to the claims as an ordered combination, **without ignoring the requirements of the individual steps**. The specific, claimed features of these rules allow for the improvement realized by the invention.

Id. at 1314. Emphasis added.

Like the claims in *McRO*, the instant claims incorporate specific, meaningful requirements that are restricted to a specific application of weighted signal measurements, namely, detecting the presence or absence of each analyte of a plurality of analytes. Specifically, the rules of the instant

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claims are analogous to the rules recited in *McRO*, as illustrated in table format below for the convenience of the Office.

Rules of the Instant Independent Claims	**Rules from *McRO***
Encoding each of said analytes as at least one first value in a first component of a signal (as recited in claim 49) or encoding each of said analytes as at least one first value and at least one second value (as recited in claim 55) to generate a coding scheme, in which the first value is an intensity or range of intensities and the second value is a wavelength or range of wavelengths;	Obtaining a first set of rules that define output morph weight set stream as a function of phoneme sequence and time of said phoneme sequence;
Providing a sample and then contacting said sample with the analyte-specific reagents to generate the first values or the first and second values;	Obtaining a timed data file of phonemes having a plurality of sub-sequences;
Cumulatively measuring the first values or the first and second values to generate cumulative measurements; and	Generating an intermediate stream of output morph weight sets and a plurality of transition parameters between two adjacent morph weight sets by evaluating said plurality of sub-sequences against said first set of rules; Generating a final stream of output morph weight sets at a desired frame rate from said intermediate stream of output morph weight sets and said plurality of transition parameters; and
Determining the presence or absence of target analytes based on the correlation between the cumulative measurements and the coding scheme, in which the method non-degenerately detects the presence or absence of at least seven analytes in a single sample volume, without immobilization of said analytes, physical separation of said analytes, or mass spectrometry.	Applying said final stream of output morph weight sets to a sequence of animated characters to produce lip synchronization and facial expression control of said animated characters.

As shown above, instant claims 49 and 55 require the rules to be rendered in a specific way: as a relationship between a non-degenerate coding scheme that comprises a first value of intensity or a first and a second values of intensity and wavelength, signal measurements, and applying a cumulative measuring approach to correlate the signal measurements to the coding scheme to determine the presence or absence of target analytes. Similarly, claim 1 of *McRO* "requires that the

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rules be rendered in a specific way: as a relationship between subsequences of phonemes, timing, and the weight to which each phoneme is expressed visually at a particular timing (as represented by the morph weight set)." *Id.* at 1315. Hence, the steps of the instant claims provide specific and meaningful limitations and further "allow for the improvement realized by the invention."

B. The Instant Invention is Directed to a "Process Designed to Solve a Technological Problem"

The Office asserts that "[t]he signal processing is a manner of mathematical manipulation analogous to the Arrhenius equation of *Diamond v. Diehr*, 450 U.S. 175 (1981), which was also found to be abstract." *See* Office Action at page 3.

Applicant respectfully submits that this is a misinterpretation of the teachings of *Diamond v. Diehr*. In *Diamond v. Diehr*, the court concluded that:

> [W]hen a claim containing a mathematical formula implements or applies that formula in a structure or process which, when considered as a whole, is performing a function which the patent laws were designed to protect (e. g., transforming or reducing an article to a different state or thing), then the claim satisfies the requirements of § 101. Because we do not view respondents' claims as an attempt to patent a mathematical formula, but rather to be drawn to an industrial process for the molding of rubber products.

Diehr, 450 U.S. at 192-193.

Key to the Court's analysis was the fact that the claim included monitoring of temperature data and concluded by reciting sending instructions to "open the press automatically when a [the calculation] indicates completion of curing." *Diehr*, 450 U.S. 175 at 180. The Court found that this anchored the use of the ineligible equation to a real-world process and the *Diehr* Court's analysis was cited with approval in *Alice*.

Similarly, the *McRO* court, regarding *Diehr*, states that "the [*Diehr*] Court looked to how the claims 'used that equation in a process designed to solve a technological problem in 'conventional industry practice.'" *Id.* at 1314, quoting *Alice*, 134 S. Ct. at 2358 (in turn quoting *Diehr*, 450 U.S. at 178).

Analogously in the instant case, the claims utilize the cumulative measurement of the first value of intensity or the first and second value of intensity and wavelength in combination with the

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non-degenerate coding scheme to solve the technological problem of determining the presence or absence of at least seven target analytes from a plurality of analytes in a single sample volume, without the need to immobilize the analytes, physically separating the analytes, or perform a mass spectrometry analysis. As in *Diehr* and *McRO*, the use of the cumulative measurement in a process designed to detect at least seven target analytes in a single sample volume anchors the claim in patent-eligibility.

C. The Claims Recite an Inventive Concept

Even if the Office asserts that claims 49 and 55 are directed to an abstract idea, which Applicant would strongly disagree with, the claims recite "significantly more" than an abstract idea itself. As noted in the previous response filed on May 2, 2017, the Nov. 2016 Eligibility Memo further provides guidance on how examiners should apply the second step. Specifically, the Nov. 2016 Eligibility Memo highlights that regarding *BASCOM*, "examiners should consider the additional elements in **combination,** as well as individually, when determining whether a claim as a whole amounts to significantly more, as this may be found in the nonconventional and non-generic arrangement of known, conventional elements." (Emphasis in Original) Furthermore, the Nov. 2016 Eligibility Memo notes that "the district court erred by failing to recognize that when combined, an inventive concept may be found in the non-conventional and non-generic arrangement of the additional elements, *i.e.,* the installation of a filtering tool at a specific location, remote from the end-users, with customizable filtering features specific to each end user (note that the term 'inventive concept' is often used by the courts to describe additional element(s) that amount to significantly more than a judicial exception)."

Like the claims of *BASCOM*, the claims of the instant invention require encoding each of the plurality of analytes with a first value of intensity or a first and second value of intensity and wavelength to generate a non-degenerate coding scheme, cumulatively measuring the intensity or intensity and wavelength of each of the plurality of analytes to generate cumulative measurements, and based on a correlation of the cumulative measurement and the coding scheme, determine the presence of target analytes. Furthermore like *BASCOM*, the cumulative measurements can only be performed within a computer, *i.e.*, a microscopic instrument capable of measuring the intensity

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and/or wavelength of the labeled analytes. As such, the claims of the instant invention, when considered as a whole, amount to significantly more than an abstract idea, and recite an inventive concept.

D. The Instant Invention Does Not Seek to Monopolize

Lastly, Applicant does not seek to preempt others from all possible uses of analyte detection by a coding scheme. The Federal Circuit, in *McRO, Inc. v. Bandai Namco Games Am. Inc.*, opined that "[t]he preemption concern arises when the claims are not directed to a specific invention and instead improperly monopolize 'the basic tools of scientific and technological work.'" *McRO, Inc. v. Bandai Namco Games Am. Inc.*, 837 F.3d 1314 (Fed. Cir. 2016) (quoting *Alice*, 134 S. Ct. at 2354, in turn quoting *Myriad*, 133 S. Ct. at 2116). The *McRO* court further explains that the claims at issue were valid under § 101 because:

> The specific structure of the claimed rules would prevent broad preemption of all rules-based means of automating lip synchronization unless the limits of the rules themselves are broad enough to cover all possible approaches. … By incorporating the specific features of the rules as claim limitations, claim 1 is limited to a specific process for automatically animating characters using particular information and techniques and does not preempt approaches that use rules of a different structure or different techniques.
>
> *Id.* at 1315-16.

Similarly, the *BASCOM* court notes that "the claims of the '606 patent do not preempt the use of the abstract idea of filtering content on the Internet or on generic computer components performing conventional activities. The claims carve out a specific location for the filtering system (a remote ISP server) and require the filtering system to give users the ability to customize filtering for their individual network accounts." *BASCOM Global Internet Services v. AT&T Mobility LLC,* 827 F .3d 1350 (Fed. Cir. 2016).

Here, as with the claims in *McRO* and *BASCOM*, the instant claims do not preempt all rules-based means of detecting target analytes by a coding scheme. Instead, the instant claims are limited to a specific process of detecting the presence or absence of each analyte of a plurality of analytes in a single sample volume by using a particular non-degenerate coding scheme in combination with

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cumulative measurement of intensity or intensity and wavelength, without immobilization, physical separation, or mass spectrometry.

Based on the above, Applicant respectfully submits that the instant claims are directed to patent eligible subject matter. Withdrawal of the rejection under 35 U.S.C. § 101 is respectfully requested.

III. Claim Rejections Under 35 U.S.C. §102

The Office had maintained the rejection to previously submitted claims 49, 51, 53, 55-56, 75-78, 80-81, 84-88, and 90-92 under pre-AIA 35 U.S.C. 102, citing a new reference, namely Lin et al. (US2013/0261019A1) (hereinafter Lin), to support the rejection. In particular, the Office asserted that Lin teaches "nondegenerate combinations of barcodes" and that the methods of Lin "do not involve immobilization of analytes, physical separation of analytes, or mass spectrometry." *See* Office Action at pp. 4-7.

Applicant respectfully traverses the rejection. Lin teaches nucleic acid barcode probes which comprise a nucleic acid nanostructure that has two or more fluorescently labeled regions to generate a specific color-coded pattern. *See*, e.g., paragraph [0004], Fig. 1, and Fig. 4A. Lin further teaches a triple color-coded barcode probe that can utilize a combination of blue (B), red (R), and green (G) colors, or a combination of B, R, G, B+G, B+R, and G+R colors. To resolve a target of interest, Lin teaches that three steps can be applied. In step one, each fluorescent spot is identified and then filtered based on threshold intensity and size criteria. In step two, the distances (calculated from (x,y) coordinates of fluorescent spots) between each region of the probes and the bending angle (based on the center spot) are calculated. Additional distance criteria between red and blue, blue and green, and red and green spots are also utilized during this step. In step three, additional criteria such as theoretical distance and color are further utilized to generate a final result on the presence or absence of the target of interest. *See* paragraph [0095].

Lin differs from the instant invention because Lin teaches a **degenerate** coding scheme. For example, consider Lin's color coding using the six colors described above, B, R, G, B+G, B+R, and G+R, color combinations can include BGB, GGB, and (B+G)GB. In a first sample volume containing an isolated first target (Target 1) labeled with BGB, one would observe three distinct

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spots, blue, green, and blue. In a second sample volume containing an isolated second target (Target 2) labeled with GGB, one would observe three distinct spots, green, green, and blue. Similarly in a third sample volume containing an isolated third target (Target 3) labeled with (B+G)GB, the three distinct spots will be cyan (B+G), green, and blue.

However, in a sample volume comprising a mixture of Target 1, Target 2, and Target 3, if Target 1 and Target 2 physically overlap with each other (*i.e.* are not spatially separated), then, based on the analysis of Lin, namely, step one of identifying each fluorescent spot and steps two and three of utilizing the pre-determined distance and angle criteria, one would not be able to distinguish the presence of Target 1 and Target 2 from Target 3. This failure of the methods taught by Lin is as a result of the codes specified being **degenerate**. To further illustrate this point for the convenience of the Office, please see Fig. 1A and Fig. 1B below.

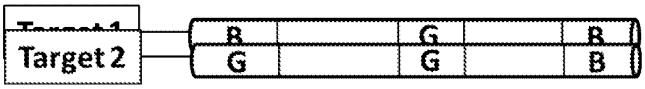


Fig. 1A

Here, B+G, green, and blue spots will be detected when Target 1 and Target 2 physically overlap, and these color spots cannot be distinguished from the B+G, green, and blue spots of Target 3. As such, the scheme used by Lin is incapable of discriminating target labels in combination. This precludes the use of the scheme taught by Lin for unambigious coding of targets.

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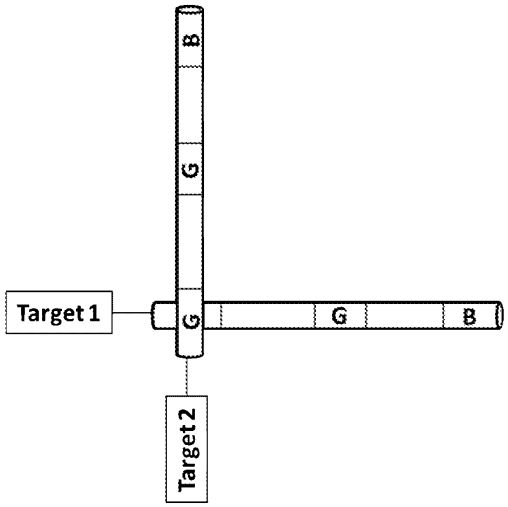


Fig. 1B

Similarly here, B+G, green, and blue spots will be detected when Target 1 and Target 2 overlap at an angle, and these color spots cannot be distinguished from the B+G, green, and blue spots of Target 3.

As set out in the MPEP, [a] claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. (MPEP 2131, quoting *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). "[U]nless a reference discloses within the four corners of the document not only all of the limitations claimed but also all of the limitations arranged or combined in the same way as recited in the claim, it cannot be said to prove prior invention of the thing claimed and, thus, cannot anticipate under 35 U.S.C. § 102." *Net MoneyIn, Inc. v. VeriSign, Inc.*, No. 2007-1565 slip op. at 18-19 (Fed. Cir. Oct. 20, 2008).

In the instant case, Lin does not teach "each and every element as set forth in the claims" because Lin teaches a degenerate coding scheme. The instant case differs because the coding scheme is non-degenerate. Therefore, the instant invention is not anticipated by Lin for at least this reason.

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Furthermore, Lin does not teach a coding scheme based on both intensity and color. Instead, the crux of Lin's invention is the use of color-coded patterns deposited on a nanorod barcode probe to detect a target of interest. The instant invention utilizes either intensity or a combination of intensity and wavelength to generate a coding scheme. Therefore, the instant invention is not anticipated by Lin also for at least this reason.

In light of the above, Applicant respectfully submits that independent claims 49 and 55 and their respective dependent claims are not anticipated by Lin. Withdrawal of the rejection under pre-AIA 35 U.S.C. 102(e) is respectfully requested.

IV. Claim Rejections Under 35 U.S.C. §103

The Office had rejected former claim 79 under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Lin in further view of Nie et al. (US 2003/0148544 A1) (hereinafter Nie). The Office further rejected former claim 83 under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Lin in further view of Orpana et al. (WO 2010/128206 Al) (hereinafter Orpana). *See* Office Action at pp. 8-11.

These rejections are respectfully traversed in view of the present claim amendments and the following arguments. MPEP 2143 states that obviousness may be established only when three basic criteria have been met. "*First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.*"

As discussed above with respect to Lin, Lin teaches a degenerate coding that is based solely on the combination of colors. Lin is silent on the use of a non-degenerate coding scheme and further does not contemplate the use of intensity or intensity and wavelength as is described in the instant invention to detect at least seven target analytes in a sample volume. Therefore, the instant invention is not obvious in view of Lin.

Nie does not cure the deficiencies of Lin. Furthermore, there is no teaching, suggestion, or motivation in the combination of Lin and Nie for a skilled artisan to arrive at the instant invention as both references do not teach or suggest a non-degenerate coding scheme. Therefore, the

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combination of Lin and Nie fails to 1) suggest or motivation a skilled artisan to modify the references or to combine the reference teachings to arrive at the instant invention; 2) there is no reasonable expectation of success because both Lin and Nie are silent on a non-degenerate coding scheme; and 3) Lin and Nie do not teach or suggest all of the claim limitations of the instant invention. In light of this, Applicant respectfully submits that claim 79 is not obvious in view of Lin and Nie.

With regard to Orpana, Orpana also does not cure the deficiencies of Lin. In addition, there is no teaching, suggestion, or motivation in the combination of Lin and Orpana for a skilled artisan to arrive at the instant invention as both references do not teach or suggest a non-degenerate coding scheme. Therefore, the combination of Lin and Orpana also fails to 1) suggest or motivation a skilled artisan to modify the references or to combine the reference teachings to arrive at the instant invention; 2) there is no reasonable expectation of success because both Lin and Orpana are silent on a non-degenerate coding scheme; and 3) Lin and Nie do not teach or suggest all of the claim limitations of the instant invention. As such, claim 83 is not obvious in view of Lin and Orpana.

In light of the above, Applicant respectfully request withdrawal of the rejection under pre-AIA 35 U.S.C. 103(a).

V. Double Patenting

Claims 49, 51, 53, 55, and 56 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 20-24, respectively, of U.S. Patent No. 8,838,394 B2 [on IDS of 5/2/2017].

Applicant respectfully traverses the rejection. However, solely to expedite prosecution, Applicant concurrently files a Terminal Disclaimer in compliance with 37 C.F.R. §1.321 with respect to U.S. Patent No. 8,838,394. This Terminal Disclaimer obviates the present rejection. Accordingly, Applicant requests that this rejection be withdrawn.

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CONCLUSION

In light of the remarks set forth above, Applicant believes that the pending claims are under condition for allowance. Applicant respectfully solicits the Examiner to expedite the prosecution of this patent application to issuance. Should the Examiner have any question, the Examiner is encouraged to telephone the undersigned.

The Commissioner is authorized to charge any underpayment or credit any overpayment to Deposit account No. 23-2415 (Attorney Docket No. 38075-716.301).

Respectfully submitted,

Date: August 11, 2017 By: /Lee A. Johnson /
Lee A. Johnson, Reg. No. 75,671

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Amendments to the Claims

This listing of claims will replace all prior versions, and listings, of claims in this application. The following amendments do not constitute an admission regarding the patentability of the amended subject matter and should not be so construed. Applicants reserve the right to pursue the subject matter of the canceled claims in this or any other appropriate patent application. Further, none of Applicants' amendments are to be construed as dedicating any such subject matter to the public, and Applicants reserve all rights to pursue any such subject matter in this or a related patent application.

Listing of claims:

1. **(Withdrawn)** An assay that is capable of unambiguously detecting the presence or absence of each of at least seven (7) analytes, in any combination of presence or absence, in a single sample volume without immobilization of said analytes, physical separation of said analytes, melting curve analysis, or mass spectrometry.
2. **(Withdrawn)** The assay of claim 1, wherein each of said analytes is encoded as a value of one component of a signal.
3. **(Canceled)**
4. **(Withdrawn)** The assay of claim 2, wherein each of said analytes is encoded as at least one first value in a first component of said signal and at least one second value in a second component of said signal.
5. **(Withdrawn)** The assay of claim 2, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

6.-8. **(Canceled)**

9. **(Withdrawn)** The assay of claim 4, wherein said first value is an intensity or range of intensities and said second value is a wavelength or range of wavelengths.

10.-12. **(Canceled)**

13. **(Withdrawn)** The assay of claim 2, wherein said signal is an electromagnetic signal.

14.-16. **(Canceled)**

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17. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with reagents comprising hybridization probes.

18. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with a plurality of hybridization probes, comprising an identical fluorophore, wherein said plurality of hybridization probes are specific for different analytes.

19. **(Withdrawn)** The assay of claim 1, wherein said detecting is performed with a plurality of hybridization probes comprising different fluorophores, wherein said plurality of hybridization probes are specific for different analytes.

20.-21. **(Canceled)**

22. **(Withdrawn)** The assay of claim 2, wherein said signal is generated during a polymerase chain reaction.

23.-26. **(Canceled)**

27. **(Withdrawn)** The assay of claim 4, further comprising constructing a diagram.

28. **(Withdrawn)** The assay of claim 27, wherein said diagram is constructed by plotting possible combinations of said first values and said second values for positive control samples.

29. **(Withdrawn)** The assay of claim 1, wherein at least one of said analytes comprises a polynucleotide.

30.-33. **(Canceled)**

34. **(Withdrawn)** The assay of claim 1, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.

35. **(Withdrawn)** The assay of claim 1, further comprising providing information concerning the presence or absence of said analyte to a health care professional, wherein the health care professional makes a clinical decision based on said information.

36. **(Canceled)**

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37. **(Withdrawn)** The assay of claim 1, wherein at least one step of said assay is performed using a computer.

38. **(Withdrawn)** The assay of claim 37, wherein said computer is located on a remote server.

39. **(Withdrawn)** The assay of claim 37, wherein said computer is located on a thermal cycler.

40.-41. **(Canceled)**

42. **(Withdrawn)** The assay of claim 2, wherein said coding is innately non-degenerate.

43. **(Withdrawn)** The assay of claim 42, wherein said coding is designed to be non-degenerate by enumerating legitimate results that can be obtained from said encoding, identifying legitimate results that are degenerate, and eliminating at least one potential analyte code from said encoding to eliminate degeneracy.

44. **(Canceled)**

45. **(Withdrawn)** The assay of claim 1, wherein said assay is an end-point assay.

46. **(Canceled)**

47. **(Withdrawn)** The assay of claim 1, wherein said assay is a liquid phase assay.

48. **(Canceled)**

49. **(Currently Amended)** A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

a)[[a.]] encoding each of said analytes as [[a]]at least one first value in a first component of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by one of said first values, wherein said first value is an intensity or range of intensities, and wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

b)[[b.]] providing a sample comprising, or potentially comprising, at least one of said analytes;

c)[[c.]] contacting said sample with analyte-specific reagents that generate said first values, as represented in said coding scheme, when each of said analytes is present;

d)[[d.]] cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and

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e)[[e.]] determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme;

wherein said method non-degenerately detects the presence or absence of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization of said analytes, physical separation of said analytes, or mass ~~spectometry~~spectrometry.

50. **(Canceled)**

51. **(Cancelled)**

52. **(Canceled)**

53. **(Previously Presented)** The method of claim 49, wherein said first value is incremented in said coding scheme to an amount greater than the sum of said preceding first values.

54. **(Canceled)**

55. **(Currently Amended)** A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

a)[[a.]] encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said first value is an intensity or range of intensities and said second value is a wavelength or range of wavelengths, and wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

b)[[b.]] providing a sample comprising, or potentially comprising, at least one of said analytes;

c)[[c.]] contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present;

d)[[d.]] cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and

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e)[[e.]] determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme[[,]];

wherein said method, without immobilization of said analytes, physical separation of said analytes, or mass spectrometry, non-degenerately detects the presence or absence of each of at least six said analytes in a single sample volume, in any combination of presence or absence, when each of said reagents generates only one second value.

56. **(Original)** The method of claim 55, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

57.-74. **(Canceled)**

75. **(Cancelled)**

76. **(Currently Amended)** The method of claim [[75]]49, ~~wherein each of said analytes is encoded as at least one first value in said first component of said signal and~~ further comprising encoding each of said analytes as at least one second value in a second component of said signal.

77. **(Currently Amended)** The method of claim [[75]]49, wherein each of said analytes is encoded as said first value in a first component of said signal and as [[at]] each of a plurality of second values in a second component of said signal.

78. **(Currently Amended)** The method of claim 76, wherein ~~said first value is an intensity or range of intensities and~~ said second value is a wavelength or range of wavelengths.

79. **(Previously Presented)** The method of claim 49, wherein said signal is an electromagnetic signal.

80. **(Previously Presented)** The method of claim 49, wherein said detecting is performed with reagents comprising hybridization probes.

81. **(Previously Presented)** The method of claim 49, wherein said detecting is performed with a plurality of hybridization probes, comprising an identical fluorophore, wherein said plurality of hybridization probes are specific for different analytes.

82. **(Withdrawn)** The method of claim 49, wherein said detecting is performed with a plurality of hybridization probes comprising different fluorophores, wherein said plurality of hybridization probes are specific for different analytes.

83. **(Previously Presented)** The method of claim 49, wherein said signal is generated during a

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polymerase chain reaction.

84. **(Previously Presented)** The method of claim 49, further comprising constructing a diagram.

85. **(Previously Presented)** The method of claim 84, wherein said diagram is constructed by plotting possible combinations of said first values and said second values for positive control samples.

86. **(Previously Presented)** The method of claim 49, wherein at least one of said analytes comprises a polynucleotide.

87. **(Previously Presented)** The method of claim 49, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.

88. **(Previously Presented)** The method of claim 49, further comprising providing information concerning the presence or absence of said analyte to a health care professional, wherein the health care professional makes a clinical decision based on said information.

89. **(Canceled)**

90. **(Previously Presented)** The method of claim 49, wherein said encoding is designed to be non-degenerate by enumerating legitimate results that can be obtained from said encoding, identifying legitimate results that are degenerate, and eliminating at least one potential analyte code from said encoding to eliminate degeneracy.

91. **(Previously Presented)** The method of claim 49, wherein said method does not comprise immobilization of said analytes.

92. **(Previously Presented)** The method of claim 55, wherein said method does not comprise immobilization of said analytes.

93. **(New)** The method of claim 49, wherein at least one step of said method is performed using a computer.

94. **(New)** The method of claim 93, wherein said computer is located on a remote server.

95. **(New)** The method of claim 93, wherein said computer is located on a thermal cycler.

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 14/451,876	Filing Date 08/05/2014	☐ To be Mailed

ENTITY: ☐ LARGE ☒ SMALL ☐ MICRO

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	RATE ($)	FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	**140**
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	**140**

APPLICATION AS AMENDED – PART II

AMENDMENT		(Column 1)		(Column 2)	(Column 3)	RATE ($)	ADDITIONAL FEE ($)
	08/11/2017	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		
	Total (37 CFR 1.16(i))	* 45	Minus	** 45	= 0	X $40 =	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	X $210 =	0
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	**0**

AMENDMENT		(Column 1)		(Column 2)	(Column 3)	RATE ($)	ADDITIONAL FEE ($)
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))						
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))						
						TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

LIE
GLORIA J. TRAMMELL

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Application Number	Application/Control No.	Applicant(s)/Patent under Reexamination	
	14/451,876	KARTALOV ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☐ APPROVED	☒ DISAPPROVED
Date Filed : 11 August, 2017	**This patent is subject to a Terminal Disclaimer**	

Approved/Disapproved by:

CHARRISSA BROWN

Technology Center: PLRC

Telephone: (571)272-1558

The disclaimer fee under 37 CFR 1.20(d) in the amount of $160.00 has not been submitted, nor is there any pre authorization in the application to charge to a deposit account. (See FP 14.24 and 14.26.07.)

The person who signed the terminal disclaimer is not the applicant, patentee or an attorney or agent of record. 37 CFR 1.321(a) and (b). (See FP 14.26.08)

Re-submit the TD along with a POA, (Power Of Attorney) with no additional fees required.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/451,876	08/05/2014	Emil Kartalov	38075-716.301	1065

21971 7590 08/23/2017

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EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1631	

NOTIFICATION DATE	DELIVERY MODE
08/23/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

Applicant's arguments filed 11 August 2017 have been fully considered but they are not persuasive.

On pages 10-14 of the Remarks, applicant goes into great detail attempting to align the fact pattern of *McRO* with the fact pattern of the instant set of claims. In *McRO*, the claims were found to be not directed to a judicial exception because the algorithm resulted in automated human animation that was previously only accomplished by humans. In addition, the algorithm of *McRO* details a technique for solving a problem in technology and not merely analysis of output data.

The instant set of claims recite a plurality of judicial exceptions. Step a of claim 49 recites encoding the analytes which is a judicial exception. Step d of claim 49 recites cumulatively measuring signal values. Step e of claim 49 recites a mental step of determining presence/absence patterns. These judicial exceptions are in combination with routine and conventional empirical steps that are not automated. The determination of the presence/absence patterns is the analysis of output data to determine a coding scheme.

On pages 13-14 of the Remarks, applicant argues that the fact pattern of the claims is analogous to the fact pattern of *Diehr*. Applicant argues that similar to *Diehr*, the cumulative measurement results in an improvement to the technology by resulting in converting the data into presence/absence patterns. This argument is not persuasive because the judicial exception of the Arrhenius equation is *Diehr* improved limitations outside the judicial exception- namely, the rubber molding process. In contrast, while

converting data into presence/absence patterns may improve the analysis of data, better data processing does not improve an element outside the judicial exceptions.

On pages 14-15 of the Remarks, applicant argues that the fact pattern of the instant claims is analogous to the fact pattern in *BASCOM*. In *BASCOM*, the combination of the limitations necessarily required execution on a computer; consequently, the claims were patent eligible. In the instant set of claims, the combination of limitations requires human intervention in the empirical steps.

Applicant argues that the limitations of the claim are specific enough as to not monopolize a technical field. This argument is not persuasive because the specificity and complexity of an idea do not preclude the idea from being abstract.

With regard to the 35 U.S.C. 102 rejection, applicant argues that Lin et al. teaches a degenerate coding scheme. Applicant argues that Line teaches blue (B), red (R), green (G), B+G, B+R, and G+R. Figure 2C of Lin et al. illustrates all 27 non-generate combinations of B, G, and R. Figure 2C of Lin et al. does not involve B+G, B+R, or G+R in the combinations. While Figures 1A and 1B of the Remarks illustrate the degenerate coding of cyan, green, and blue; Figure 2C of Lin et al. does not teach cyan as one of the three colors. While Figure 5D of Lin et al. teaches dual-labeled zone fluorescence coding that would result in degeneracy, Figure 2C of Lin et al. teaches single-zone fluorescent coding that is non-degenerate. In the instant 35 U.S.C. 102 rejection, only single-labeled-zone fluorescent barcodes teachings of Lin et al. are relied upon. In other words, an observer witnessing a spot that is cyan, green, and blue should deduce that the spot is the non-degenerate overlap of BGB and GGB.

Applicant argues that Lin et al. does not teach the amended limitations of the claims. This argument is not persuasive because the proposed amendments have not been entered.

With regard to the 35 U.S.C. 103 rejection, applicant argues that Nie et al. and Orpana et al. do not overcome the alleged deficiencies of Lin et al. These arguments are not persuasive because the document of Lin et al. is not deficient.

With regard to the double patenting rejection, the Terminal Disclaimer is being processed.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 10:30 am to 5 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Peter Paras, Supervisory Patent Examiner, can be reached at (571) 272-4517.

Information regarding the status of the application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only.

/Russell S. Negin/
Primary Examiner, Art Unit 1631
20 August 2017